THE ROLE OF PROTEIN KINASE C IN ANTERIOR PITUITARY HORMONE RELEASE

by

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This thesis is dedicated to my parents whose support and encouragement have made this possible.

I declare that the studies presented in this Thesis are the result of my own independent investigation with the exception of the radioimmunoassays, which were carried out with the assistance of John Bennie and Sheena Carroll, and some of the PKC activity assays, which were carried out with the assistance of Melanie Johnson.

This work has not been and is not being currently submitted for candidature for any other degree.

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ABBREVIATIONS

AA	=	arachidonic acid
AVP	=	arginine vasopressin
BrPheBr	=	ρ-bromophenacyl bromide
BSA	=	bovine serum albumin
DAG	=	diacylglycerol
DAG lipase	=	diacylglycerol lipase
DMEM	=	Dulbecco's modified Eagle's medium
DMF	=	dimethylformamide
DMS	=	dimethylstaurosporine
DOG	=	1,2-dioctanoyl-*sn*-glycerol
DOPPA	=	12-deoxyphorbol 13-phenylacetate 20-acetate
E_2	=	oestrogen
E64	=	trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
EDTA	=	ethylenediaminetetraacetic acid
EGTA	=	ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
EtSH	=	2-mercaptoethanol
ETYA	=	5,8,11,14-eicosatetraynoic acid
FSH	=	follicle-stimulating hormone
G protein	=	guanine nucleotide-binding protein
GDP	=	guanosine diphosphate
GH	=	growth hormone
GHRH	=	growth hormone-releasing hormone
GTP	=	guanosine triphosphate
H7	=	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETE	=	hydroxyeicosatetraenoic acid
HPETE	=	hydroperoxyeicosatetraenoic acid
Ins(1,4,5)P_3	=	inositol 1,4,5-trisphosphate
LH	=	luteinizing hormone
LHRH	=	luteinizing hormone-releasing hormone
LT	=	leukotriene
MAG lipase	=	monoacylglycerol lipase
MEM	=	minimal essential medium
NDGA	=	nordihydroguaiaretic acid
ODS	=	octadecyl silica
ONO-RS-082	=	4-chloro-*N*-(ρ-pentylcinnamoyl) anthraniric acid
PAF	=	platelet-activating factor
PDBu	=	phorbol 12,13-dibutyrate
PDGF	=	platelet-derived growth factor
PG	=	prostaglandin
PI-3kinase	=	phosphatidylinositide 3-kinase
PKA	=	cAMP-dependent protein kinase
PKC	=	protein kinase C

PKG	=	cGMP-dependent protein kinase
PKM	=	protein kinase M
PLA_2	=	phospholipase A_2
PLAP	=	phospholipase A_2-activating protein
PLC	=	phospholipase C
PLD	=	phospholipase D
PMA	=	phorbol 12-myristate 13-acetate
PMSF	=	phenylmethylsulphonyl fluoride
PPH	=	phosphatidate phosphohydrolase
PtdCho	=	phosphatidylcholine
PtdIns	=	phosphatidylinositol
PtdIns(3,4)P_2	=	phosphatidylinositol 3,4-biphosphate
PtdIns(3,4,5)P_3	=	phosphatidylinositol 3,4,5-trisphosphate
PtdIns(4,5)P_2	=	phosphatidylinositol 4,5-biphosphate
PtdOH	=	phosphatidate
RHC 80267	=	1,6-di(*O*-(carbamoyl)cyclohexaneoxime)hexane
TK	=	tyrosine kinase
TRH	=	thyrotrophin-releasing hormone
TX	=	thromboxane

ABSTRACT

The role of protein kinase C (PKC) in luteinizing hormone-releasing hormone (LHRH)-induced luteinizing hormone (LH) release and in the priming effect of LHRH (the unique ability of LHRH to increase pituitary responsiveness to itself) was examined. Using pro-oestrous rat anterior pituitary pieces, incubated *in vitro,* the PKC inhibitors, staurosporine, Ro 31-8220 and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) inhibited the induction of LHRH priming, but had no effect on initial, unprimed LHRH-induced release of LH. Although the primed response was readily inhibited by staurosporine and Ro 31-8220, it was unusually resistant to block by H7, suggesting that the induction of LHRH priming may involve activation of an H7-resistant form of PKC or PKC-like kinase.

Since the PKC(s) which control LH release may have a distinct pharmacological profile, the effect of PKC inhibitors and activators (phorbol 12,13-dibutyrate (PDBu) 1,2-dioctanoyl-*sn*-glycerol (DOG) and mezerein) on hormone release was examined in more detail. Mezerein and PDBu both induced LH and growth hormone (GH) release, whereas LH, but not GH, release could be induced by DOG. Thus DOG may activate at least some of the PKC forms which induce LH release, but not those which induce GH release. Although PKC activator-induced LH and GH release were readily inhibited by staurosporine, PDBu- and mezerein-induced GH release were resistant to block by H7. Furthermore, PKC activator-induced LH release displayed both H7-sensitive and H7-resistant components. Cytosolic PKC activity, partially purified from anterior pituitary, was measured in a mixed micelle assay. A PDBu-induced, Ca^{2+}-independent, histone kinase activity was detected which was relatively resistant to H7. In contrast, Ca^{2+}-dependent PKC activity displayed the expected sensitivity to H7. Therefore, the H7-resistant PKCs which have a role in LHRH priming, LH release and GH release may be of the Ca^{2+}-independent type. Further attempts to characterise the H7-resistant kinase(s) by H7 displacement of [^{3}H]-dimethylstaurosporine binding to sites in different tissues were

made, but only limited conclusions could be drawn since the binding sites on PKC for these two inhibitors appear to be non-identical.

The cellular targets of the PKCs which control anterior pituitary hormone release were investigated. The induction of LHRH priming, but not LHRH-induced LH release from unprimed tissue, was inhibited by a number of inhibitors of phospholipase A_2 (PLA_2). Phorbol 12,13-dibutyrate-induced LH release was inhibited by PLA_2 inhibitors, suggesting that PLA_2 is a target (not necessarily directly) for the PKC(s) which control LH release. Consistent with this hypothesis, PDBu elicited [^{3}H]-arachidonic acid (AA) release from prelabelled pituitary tissue, a response which showed partial H7-resistance. Furthermore, LHRH-induced [^{3}H]-AA release was relatively H7-resistant, suggesting that the form of PKC responsible for induction of LHRH priming can activate PLA_2. Additional studies indicated that PKC modulation of PLA_2 activity was dependent upon protein synthesis. In contrast, the PKC(s) which induced GH release did not appear to do so by a mechanism involving PLA_2.

Investigations into the steroidal control of PKCs and their influences in anterior pituitary tissue showed that the expression and actions of certain PKC forms in gonadotrophs measured, *in vitro*, can be modulated by prior treatment with oestradiol-17β (E_2), *in vivo*. Oestrogen treatment of ovariectomised rats enhanced tissue responsiveness to LHRH and PDBu-induced LH, but not GH release measured *in vitro*. This action of E_2 seemed to involve synthesis of addition kinases. The E_2-induced increase in gonadotroph responsiveness (in particular the magnitude of the LHRH priming effect) thus appears to involve LHRH receptor stimulus-secretion coupling and, in particular, a facilitation of the increased expression of the PKC form(s) that are crucial for the induction of priming.

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CHAPTER 1

INTRODUCTION

1.1 SIGNAL TRANSDUCTION THROUGH PHOSPHOLIPID HYDROLYSIS

Experiments performed by Hokin and Hokin (1953) showed that carbachol stimulation of pigeon pancreatic slices enhanced the incorporation of ^{32}P orthophosphate into the head groups of the membrane phospholipids, phosphatidylinositol (PtdIns), and its metabolic precursor, phosphatidate (PtdOH). These experiments pioneered a number of studies which lead to the hypothesis that the stimulation of certain cell surface receptors initiates PtdIns breakdown, and that this action is important for the control of physiological responses such as cell division, secretion, contraction and neural activity (Michell, 1975).

More recently, compelling evidence for receptor-induced breakdown of phosphatidylcholine (PtdCho) and phosphatidylethanolamine has also been provided and this mechanism has also been suggested to have a physiological role (see Löffelholz, 1989; Billah and Anthes, 1990; Exton, 1990 for reviews).

1.1.1 Receptor controlled phosphoinositide hydrolysis

Receptor-mediated breakdown of inositol-containing phospholipids occurs mainly by the catalytic action of the phospholipase C (PLC) enzyme family (Figure 1.1). A number of reports have suggested that the polyphosphoinositides, rather than PtdIns, are the preferred substrates of PLC. For example, in experiments performed by Irvine and co-workers (1984), a mixture of brain PLCs was incubated with a lipid mixture which had inositol lipids as a minor component, similar to the composition of the inner leaflet of the plasma membrane. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) was hydrolysed 100 times more rapidly than PtdIns suggesting that PtdIns (4,5)P_2 is the preferred substrate. Immunological analysis of purified proteins, biochemical investigations and molecular cloning studies have revealed that PLC exists as several different classes of isoform (α, β, γ and δ) and that each class has several related members (see Meldrum *et al,* 1991 for review). However, recent studies suggest that the putative PLCα cDNA encodes a protein

which is devoid of PLC activity (Srivastava *et al,* 1991). Nevertheless, it is possible that the authentic β, γ and δ PLC isoforms have distinct properties, are differentially regulated and that such differential control is of physiological importance.

Receptor-induced activation of PLC can be mediated via the actions of guanine nucleotide-binding proteins (G-proteins). In general, receptor-regulated G-proteins are heterotrimeric structures, consisting of α, β and γ subunits, which transduce signals from agonist-occupied receptors to their effector enzymes in a GTP-dependent manner. For example, in permeabilized rat mast cells, the non-hydrolysable analogue of GTP, GTP-γ-S (which causes prolonged G-protein activation), induced both histamine release and inositol phospholipid hydrolysis (Cockcroft and Gomperts, 1985; Cockcroft *et al,* 1987) indicating G-protein involvement in agonist-induced histamine release. Further evidence for G-protein control of PLC activity has come from additional studies which showed that GTP could modulate agonist binding to PLC-linked receptors and that GTP could activate PLC in plasma membranes from blowfly salivary glands (Litosch *et al,* 1985). A considerable number of G-protein α subunits have been identified (see Simon *et al,* 1991 for review), some of which have been implicated in the control of PLC activity. The majority of receptors which couple to PLC do so via pertussis toxin-insensitive G-proteins, including, for example, the platelet thromboxane A_2 receptor which may couple to certain members of the G_q class (Shenker *et al,* 1991). Interestingly, a G_q protein from bovine liver selectively activates the β_I, but not the γ or δ forms of PLC (Taylor *et al,* 1991), suggesting that these multiple forms of G-protein may selectively control the coupling of receptors to specific isoforms of PLC.

It is clear that G-proteins are not the sole means by which receptors can couple to PLC. In Swiss 3T3 cells, platelet-derived growth factor (PDGF) can activate the intrinsic tyrosine kinase (TK) activity of the receptor and induce inositol phosphate production (Berridge *et al,* 1984). However, PDGF-induced inositol phospholipid hydrolysis is not modulated by GTP analogues (Cattaneo and

Vincentini, 1989), suggesting that PLC activation may not be a G-protein dependent process. Mutant growth factor receptors which are defective in TK activity fail to elicit inositol phospholipid turnover (Moolenaar *et al,* 1988) and TK inhibitors prevent inositol phosphate production by some agonists (Margolis *et al,* 1989). Thus, agonist stimulation of certain growth factor receptors may activate PLC in a TK-dependent manner. The γ isoform of PLC can directly associate with the epidermal growth factor receptor and becomes phosphorylated on specific tyrosine residues, an effect which correlates with activation of the enzyme (Margolis *et al,* 1989; Meisenhelder *et al,* 1989). However, IgE receptor-induced tyrosine phosphorylation and activation of PLCγ in rat basophilic leukaemia cells appears to involve a non-receptor TK (Park *et al,* 1991). Thus, multiple mechanisms exist for the receptor control of PLC activity, each of which may selectively control the involvement of certain PLC forms in receptor responses.

Phospholipase C-catalysed hydrolysis of PtdIns(4,5)P_2 generates two products, inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (DAG), both of which serve as second messengers: Ins(1,4,5)P_3 mobilises Ca^{2+} from intracellular pools (Streb *et al,* 1983) and DAG activates the phospholipid-dependent serine/threonine-specific kinase, protein kinase C (PKC) (Nishizuka, 1984) (see section 1.2).

An important cellular function for Ins(1,4,5)P_3 was implied by early studies which showed that activation of Ca^{2+}-mobilising receptors was accompanied by the rapid formation of Ins(1,4,5)P_3 (Berridge, 1983) and that a specific Ins(1,4,5)P_3 5-phosphatase existed (Downes *et al,* 1982). The Ca^{2+} mobilising action of Ins(1,4,5)P_3 was then demonstrated by adding Ins(1,4,5)P_3 to permeabilized pancreatic acinar cells, which caused rapid Ca^{2+} release (Streb *et al,* 1983). Following Ca^{2+} release, Ca^{2+} is resequestered, and this coincides with the rapid degradation of Ins(1,4,5)P_3 (Joseph *et al,* 1984). Since these early experiments, the Ca^{2+} mobilising action of Ins(1,4,5)P_3 has been demonstrated in a number of

different cell types including anterior pituitary GH_3 cells (Berridge and Irvine, 1984). Inositol 1,4,5-trisphosphate releases Ca^{2+} from non-mitochondrial Ca^{2+} pools (probably the endoplasmic reticulum) by binding to specific receptors (Berridge and Irvine, 1989). However, a proportion of the intracellular Ca^{2+} pool (approximately 50 - 70%) is insensitive to Ins(1,4,5)P_3. The Ins(1,4,5)P_3-insensitive pools may contribute to the Ins(1,4,5)P_3-induced Ca^{2+} signal by acting as a store of Ca^{2+} which can refill depleted Ins(1,4,5)P_3-sensitive pool in a GTP-dependent manner (Ghosh *et al,* 1989).

A large number of different inositol phosphates have been identified in agonist-stimulated eukaryotic cells and putative physiological roles for each have been sought. Most mono- and bis-inositol phosphates appear to be products of Ins(1,4,5)P_3 degradation and have no known second messenger function. The levels of inositol pentakisphosphates and inositol hexakisphosphates change very slowly during agonist stimulation suggesting that they do not have an acute second messenger role (Tilly *et al,* 1987). Inositol 1,3,4,5-tetrakisphosphate has been suggested to enhance Ins(1,4,5)P_3 responses by interacting with specific intracellular recognition sites to control the transfer of Ca^{2+} between different intracellular pools (Irvine, 1990).

A number of novel inositol phospholipids have been identified which are formed during stimulation of certain receptors. For example, in thrombin-stimulated platelets, there is a time dependent production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, probably due to the activity of a phosphatidylinositide 3-kinase (PI-3 kinase) (Kucera and Rittenhouse, 1990). A physiological role for these novel inositol-containing phospholipids has yet to be elucidated, but an involvement of growth factor TK receptors in this potential signal transduction pathway is implicated by the co-association of these receptors with the Src homology-2 (SH2) domains of the p85 subunit of PI-3 kinase (Heldin, 1991).

1.1.2 Receptor-controlled phosphatidylcholine hydrolysis

During stimulation of certain agonists there is a major quantitative discrepancy between the level of $PtdInsP_2$ breakdown and DAG accumulation. For example, in hepatocytes, Ca^{2+}-mobilising agonists elicited a rapid, transient release of $Ins(1,4,5)P_3$, but a sustained accumulation of DAG (Bocckino *et al,* 1985; Charest *et al,* 1985). Determination of the fatty acid species in the DAG by HPLC showed a higher content of palmitic, oleic and linoleic acids than would have been expected for the breakdown of $PtdInsP_2$ and suggested that some of this DAG probably came from the hydrolysis of PtdCho. Direct evidence for agonist-induced PtdCho breakdown has come from experiments in which cells were pre-labelled with either [^{3}H]-choline or 1-*O*-[^{3}H] alkyl-2-lyso-*sn*-glycero-3-phosphocholine. Analysis of the labelled products of agonist stimulation indicate that PtdCho hydrolysis is probably mediated by either a PtdCho-specific PLC, which is distinct from the PtdIns-specific enzyme (Irving and Exton, 1987), or by phospholipase D (PLD) (Cabot *et al,* 1988; Pai *et al,* 1988). The action of both phospholipases liberate DAG from PtdCho; the PLC catalysed reaction directly releases DAG, whereas the actions of PLD liberates PtdOH (Figure 1.1) which can be further metabolised to DAG by the action of phosphatidate phosphohydrolase (PPH). Not only DAG but also PtdOH appears to have a number of cellular actions which may be of functional significance (see below).

In a series of studies, similar to those used to examine G-protein involvement in receptor-induced inositol phospholipid hydrolysis, agonist-induced PtdCho hydrolysis by both PLC and PLD was shown to be dependent upon G-protein action (Irving and Exton, 1987). In some cell types, agonist-induced PtdCho hydrolysis by means of PLD can be blocked by PKC inhibitors and mimicked by PKC activators, suggesting that receptor-controlled PLD activation can occur by a PKC-dependent route (Exton, 1990). Therefore, PLC (and the activation of PKC by means of the DAG it produces) may be involved in the control of PLD action.

However, some receptor types may couple to PLD, independently of PLC activation. For example, stimulation of PLD activity in rat-1 fibroblasts expressing the platelet α_2 adrenergic receptor is inhibited by pertussis toxin but is independent of inositol phosphate production and is unaffected by PKC inhibitors (MacNulty *et al,* 1992). Thus, some receptors may couple to PLD by directly interacting with G-proteins, although further studies are required to determine if this mechanism of PLD control is of physiological relevance. Activation of PLD by fMet-Leu-Phe in cytochalasin-primed neutrophils is apparently independent of PtdInsP_2-specific PLC and instead may require tyrosine phosphorylation of presently unknown targets (Uings *et al,* 1992). Therefore, certain parallels exist in the mechanism of receptors coupling to the phospholipases which catalyse PtdIns(4,5)P_2 and PtdCho hydrolysis.

Agonist-induced PtdCho breakdown provides an alternative route for the production of species of DAG which can effectively activate PKC *in vitro* (Go *et al,* 1987) and may therefore have important functional effects. If an agonist induces PtdCho breakdown, independently of inositol phospholipid hydrolysis, DAG levels will increase, but in the absence of Ins(1,4,5)P_3-induced Ca^{2+} mobilisation. Since PKC isoforms show differences in their activation profiles with respect to various DAGs and Ca^{2+} (1.2.1 and 1.2.2), it is possible that PtdCho hydrolysis may promote activation of specific PKC isoforms. Alternatively, receptor-mediated PtdCho hydrolysis may occur in a sequence involving PLC-action and PKC activation, causing a sustained release of DAG, first from the breakdown of PtdIns(4,5)P_2 and later from PLD-catalysed PtdCho hydrolysis, which may promote prolonged PKC activation. In this process, PLD could potentially have effects on its own activity by inducing DAG release which can have positive feedback actions on PKC. Such control of PLD activity has yet to be demonstrated but could contribute to bringing about sustained activation of PLD which may be important for long-term physiological and pathophysiological states. However, since the types of DAG released by PtdCho hydrolysis might activate PKC forms which are distinct from

those activated by PtdIns(4,5)P_2-derived DAG, it is conceivable that such feedback effects may not be permitted. Diacylglycerol has a number of putative cellular actions, in addition to activating PKC. In crude tissue homogenates and in reconstituted enzyme-membrane models, DAG can induce translocation of DAG kinase from the cytosol to the cell membrane (Besterman *et al,* 1986). Furthermore, certain species of DAG are enriched with arachidonic acid (AA), which has biological activity (section 1.3.5). Phosphatidic acid also has a number of biological actions and, in human carcinoma cells, can mobilise Ca^{2+} from intracellular stores (Moolenaar *et al,* 1986), and may induce inositol phospholipid hydrolysis *in vitro* by activating a PtdIns(4,5)P_2-specific PLC (Jackowski and Rock, 1989). Phosphatidic acid-dependent phosphorylation of certain proteins in extracts from rat liver, brain, lung and testis has been observed, suggesting that a phosphatidic acid-dependent protein kinase may exist (Bocckino *et al,* 1991). Any combination of these cellular actions of DAG and PtdOH may be important for the functional actions of PtdCho-metabolising receptors.

1.2 CELLULAR TARGETS AND ACTIONS OF PROTEIN KINASE C

Protein kinase C was first identified in 1977 by Nishizuka's group as a proteolytically activated protein kinase, present in many different mammalian tissues which, at that time, had no obvious role in signal transduction (Inoue *et al,* 1977). Further experiments showed that PKC was a Ca^{2+}-activated, phospholipid-dependent enzyme (Takai *et al,* 1979) and that DAG, one of the products of PLC and PLD-catalysed phospholipid hydrolysis, increased the affinity of the enzyme for Ca^{2+}, thereby activating it (Kishimoto *et al,* 1980). These studies linked PKC activation to signal transduction mechanisms. Since these early studies, PKC has been shown to exist as a family of different enzymes which have a role in many different aspects of cellular function, including secretion and exocytosis, cell proliferation, gene

expression, smooth muscle contraction, ion channel function and regulation of other aspects of signal transduction systems (Nishizuka, 1984, 1988).

1.2.1 Protein kinase C isoenzymes

Screening of various complementary DNA (cDNA) libraries has shown that PKC exists of a family of at least 10 isoenzymes which are encoded by separate genes (α, γ, δ, ε, ζ, η, λ and θ isoforms) or are splice variants of a single mRNA transcript (β_I and β_{II} isoforms) (Nishizuka, 1988; Parker *et al,* 1989; Osada *et al,* 1990; Y Nishizuka, personal communication). The α, β, and γ (group A) PKC isoform cDNA sequences have five variable regions (V_1- V_5) and four conserved regions (C_1 - C_4) (Figure 1.2) and are responsive to Ca^{2+}, phospholipid (particularly phosphatidylserine), DAG and phorbol esters. The carboxy terminal half of the enzyme contains the C_3 and C_4 regions, constitutes the catalytic domain and has sequence similarity with other protein kinases. The C_3 region of each enzyme has a putative ATP binding sequence. Interestingly, the C_4 region of the α and β but not the γ isoform has a second putative ATP-binding sequence, the significance of which is unknown. The amino terminal C_1 and C_2 regions constitute the regulatory domain, and are proposed to contain the Ca^{2+}, DAG and phospholipid binding sites and a pseudosubstrate region. Transfection of COS cells with cDNA encoding the A series isoforms show that the α, β_I/β_{II} and γ gene products correspond to the III, II and I subtypes, respectively, which are separated biochemically by hydroxyapatite column chromatography (Kikkawa *et al,* 1987). The δ, ε, ζ, η and the recently identified λ and θ (group B) PKC isoforms do not contain the C_2 region, and since these PKCs do not show Ca^{2+} dependence, this region has been suggested to contain the Ca^{2+} binding site. The ζ and λ isoforms contain only one of two cysteine-rich tandem sequences present in the C_1 region of the N-terminal regulatory domain. Since these cysteine-rich sequences are suggested to be essential for DAG/phorbol ester binding,

it is perhaps not surprising that the ζ and λ isoforms are shown to be unresponsive to phorbol esters (Liyanage *et al,* 1992; Y. Nishizuka, personal communication).

Northern blot analysis with specific oligonucleotide probes, *in situ* mRNA hybridisation techniques and immunohistochemical studies using antibodies specific to certain PKC isoforms have shown that each isoform has a distinct tissue distribution. The α, β and δ PKC isoforms have been identified in many cells and tissues examined so far, whereas the γ isoform is located in the CNS only (Nishizuka, 1988; Ogita *et al,* 1992). The ε isoform is expressed predominantly in brain tissue, with only trace amounts in the heart, lung, spleen, thymus and testis (Koide *et al,* 1992) and high levels of η PKC are detected in the skin and lung (Osada *et al,* 1990). The θ isoform is found in skeletal muscle and PKC-λ is located in the testis and ovary (Y. Nishizuka, personal communication). Many cell types contain more than one PKC isoform, and these may have different subcellular localisations (Nishizuka, 1988). This raises the possibility that each form of PKC has a distinct physiological function and that, within a cell, targets may exist which are specifically phosphorylated by different isoenzymes.

1.2.2 Activation of protein kinase C

When PKC is extracted from rat brain in the presence of Ca^{2+} chelators, it is found mainly in the soluble fraction, in an inactive form (Kikkawa *et al,* 1982). The inactive state of the enzyme may be conferred by the interaction of the pseudosubstrate sequence with the protein substrate binding site (Bell and Burns, 1991). During hormone or PKC activator stimulation, PKC is translocated to the membrane fraction in a Ca^{2+}-dependent manner (for the A series isoforms at least) where it is activated; a process which requires *sn*-1,2 DAG, Mg^{2+} ions, anionic phospholipid and ATP (Kishimoto *et al,* 1980; Nishizuka 1984, 1988; Huang, 1989).

Studies of PKC activity in mixed micelle assay systems have led to a proposed model of PKC activation for the A series PKC isoforms (Burns and Bell,

1991). In this model, soluble, inactive PKC binds Ca^{2+} and, as a result, becomes membrane associated, interacting with anionic phospholipids such as phosphatidylserine but still has relatively low kinase activity (Bazzi and Nelsestuen, 1988). The activity of this translocated PKC form is increased by phorbol esters or DAGs, which probably induce a conformational change in the enzyme, perhaps by dislocating the pseudosubstrate site from the active site. Once activated, PKC can phosphorylate itself, increasing its sensitivity to proteolytic breakdown (Ohno *et al,* 1990). Initially, the fully active catalytic subunit, protein kinase M (PKM) is released (Nishizuka, 1984), but further proteolysis at a number of different sites in the protein, causes the complete loss of activity (Schaap *et al,* 1990). Autophosphorylation and proteolysis of PKC may have an important role in the termination of PKC action.

Since the B series PKC isoforms do not translocate in response to changes in Ca^{2+}, they must be activated by a mechanism which differs from the one described above. Most of the δ isoform is suggested to be membrane associated in unstimulated cells (Ogita *et al,* 1992), indicating that an initial, Ca^{2+}-dependent translocation step is unnecessary. Instead, the activity of the already membrane bound δ isoform can be altered by phosphatidylserine and DAG alone (Ogita *et al,* 1992). The mechanism by which the cytosolic Ca^{2+}-independent PKC forms are encouraged to translocate is less clear.

The PKC isoforms differ in their susceptibility to DAG; the β form is highly DAG-sensitive, whereas the α isoform is less susceptible to DAG activation and γ PKC is relatively DAG-resistant (Naor *et al,* 1988). In addition to the effects of DAG on PKC activity, it is now clear that arachidonic acid (AA) can activate the PKC isoforms, but to different extents under different conditions of Ca^{2+} and DAG. The α isoform is activated by high concentrations of AA in the presence of Ca^{2+}, whereas, the β_I and β_{II} isoforms respond poorly to AA (Sekiguchi *et al,* 1987). However, purified β_{II}, prepared by expression in baculovirus, is maximally activated by micromolar concentrations of AA in the absence of Ca^{2+}, phosphatidylserine and

DAG (Burns *et al,* 1990). The γPKC isoform is activated by low, possibly physiological concentrations of AA (~ 10 μM), in the absence of Ca^{2+}, phosphatidylserine and DAG (Naor *et al,* 1988). More recent evidence shows that DAG can act synergistically with relatively low concentrations of AA (20 - 50 μM) to activate purified α, β and γ isoforms such that the activity of each was nearly maximal at nearly basal levels of Ca^{2+} (Shinomura *et al,* 1991). The effect of AA on the activity of the B series isoforms has yet to be studied in detail. A recent report shows that, in the presence of phosphatidylserine and DAG, AA can markedly activate ε PKC, expressed and purified from COS cells, but has inhibitory effects on δ isoform activity under the same conditions (Koide *et al,* 1992). Other cis-unsaturated fatty acids can also alter PKC activity, but are less active than AA (Sekiguchi *et al,* 1987). The biochemical mechanism of AA activation of PKC is poorly understood, but may involve an allosteric action on a site other than the DAG binding site.

It is clear that the activity of each PKC isoform can be under complex control by a number of different intracellular factors. Since different PKC isoforms have differing sensitivities for various species of DAG and cis-unsaturated fatty acids and Ca^{2+}, it is possible that the activity of each may be controlled with a degree of selectivity. For example, during PLC-catalysed inositol phospholipid hydrolysis, those isoforms which are readily affected by DAG and Ca^{2+}, i.e. the α and β forms, may be activated. Diacylglycerol released from routes that are not accompanied by intracellular Ca^{2+} mobilisation, for example, by PLD and PtdCho-specific PLC pathways, may preferentially activate the B series PKC isoforms. Arachidonic acid, which can be released by either the phospholipase A_2 (PLA_2) pathway (section 1.3) or by the sequential actions of PLC/DAG lipase, may preferentially activate the γ isoform, but inhibit the δ isoform, or in the additional presence of DAG, may preferentially activate PKC-ε. Since there is evidence that certain receptors can modulate PLA_2 action, independently of inositol phospholipid turnover (section 1.3.2.3) some agonists may preferentially activate the γ, over the β or α isoforms.

Alternatively, if PLC and PLA_2 are activated in sequence, then the initial release of DAG and Ca^{2+} may, at first, activate the α and β PKCs whilst the later release of AA may activate the γ form in a Ca^{2+}-independent manner (Naor *et al,* 1988). This action may be important for the later phase of hormone action where Ca^{2+} levels are decreasing and may be only slightly above basal. Such sustained PKC activation may be necessary for transcriptional activation and cell proliferation (Berry and Nishizuka, 1990).

1.2.3 Cellular actions of protein kinase C

A number of different cellular responses may be mediated by PKC activation, for example, PKC may have a crucial role in the feedback regulation of the Ca^{2+} mobilising pathway. In the GH_3 clonal pituitary cell line, phorbol ester pre-incubation decreases basal and thyrotrophin-releasing hormone-induced inositol phospholipid turnover (Drummond, 1985) suggesting that PKC may have negative effects on the mechanisms involved in PtdIns(4,5)P_2 hydrolysis. It is also possible that PKC may attenuate the Ca^{2+} signal by altering the activity of an IP_3 phosphatase leading to increased Ins(1,4,5)P_3 degradation, or by activating the Ca^{2+} extrusion apparatus (see Shearman *et al,* 1989b for review). The Ins(1,4,5)P_3 receptor can be phosphorylated by PKC *in vitro* (Ferris *et al,* 1991) and although the functional consequence of this action has still to be determined, this would provide an additional mechanism by which Ins(1,4,5)P_3 responses could be modulated. In thymocytes, however, phorbol esters can enhance inositol phospholipid turnover (Taylor *et al,* 1984), indicating that PKC can also have positive effects on the inositol phospholipid/Ca^{2+} signal. Protein kinase C activation can also have positive effects on other signal transduction pathways. For example, PKC can enhance PLA_2 activity, by acting synergistically with Ca^{2+} in some systems (1.3.2.2), and can modulate PLD action (1.2.2).

A number of receptor and membrane proteins, contractile and cytoskeletal proteins and are substrates for PKC *in vitro* (Nishizuka, 1984, 1988). Phosphorylation of one or more of these substrates *in vivo* may have important functional consequences. For example, phorbol 12,13-dibutyrate (PDBu) treatment of rat hippocampal pyramidal cells can increase Ca^{2+} currents in these cells (Baraban *et al,* 1985); an effect which may be due to direct phosphorylation of the ion channel (Campbell *et al,* 1988). This action of PKC on pyramidal cell channel activity may be functionally important since PKC activation in these cells can elicit some of the characteristics of long-term potentiation (Hu *et al,* 1987).

When measured in cell-free assays, each PKC isoform shows different activities towards various exogenous substrates. The A series, but not the B series, isoforms can readily phosphorylate histone, myelin basic protein and protamine (Koide *et al,* 1992; Liyanage *et al,* 1992). Therefore, *in vivo*, different isoforms may phosphorylate distinct cellular targets and may have specific physiological actions. A number of studies have shown that distinct PKC isoforms can control or influence different cellular processes. For example, when purified rat brain PKC was introduced into permeabilized cultured anterior pituitary cells, the α and β, but not the γ, isoform could induce luteinizing hormone release (Naor *et al,* 1989). In SH-Y5Y neuroblastoma cells, down regulation of certain PKC isoforms is associated with cell differentiation. Antibodies for α, β, γ, δ and ε PKCs were introduced into permeabilized SH-Y5Y cells, but only those targeted against the α and ε forms could induce cell differentiation, suggesting that α and ε PKCs are involved in this process (Leli *et al,* 1992). Further studies using antibodies specific to certain isoforms, together with genetic manipulation of the sequences encoding certain isoforms, may help to elucidate the role of PKC isoforms in cellular responses in the future.

1.2.4 Pharmacological regulation of protein kinase C activity

1.2.4.1 Protein kinase C activators

Both 1,2-DAGs and the tumour-promoting phorbol esters can directly activate PKC in cell-free assays and in intact cells by increasing the affinity of the enzyme for Ca^{2+} and/or phospholipid (Castagna *et al,* 1982; Ogita *et al,* 1992). The phorbol ester receptor co-purifies with PKC (Niedel *et al,* 1983), providing additional evidence that these agents act on PKC. Furthermore, 1,2-DAGs displace the binding of 12,13-phorbol esters to PKC (Leach *et al,* 1983), suggesting that phorbol esters mimic DAG by activating PKC at the DAG binding site.

It is now clear that there are a number of differences in the biological effects of various DAGs, phorbol esters and phorbol ester-related compounds. These differences may relate to the ability of some of these compounds to selectively activate certain PKC isoforms. The synthetic DAG, 1,2-dioctanoyl *sn*-glycerol (DOG), can mimic only some of the modulatory actions of phorbol esters on heart and anterior pituitary 'L'-type Ca^{2+} channel activity (Lacerda *et al,* 1988; MacEwan and Mitchell, 1991). Furthermore, phorbol ester-stimulated interleukin 1β mRNA production and protein phosphorylation patterns in human leukaemia cells were only partially mimicked by DOG (Strulovici *et al,* 1989). The ability of DOG to mimic only some of the actions of phorbol esters may indicate that DOG is a selective activator of distinct PKC forms. Certainly, purified PKCγ is more sensitive to DOG than the α isoform (Sekiguchi *et al,* 1988) and there is evidence that DOG has relatively low affinity for displacement of [^{3}H]-PDBu binding to α-PKC in comparison to the β and γ isoforms (MacEwan *et al,* 1992a). The actions of DOG on the activity of other PKC isoforms merits further investigation. The phorbol ester-related diterpene, mezerein, can mimic only some of the tumour-promoting actions of phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (Slaga *et al,* 1980). Jaken and her co-workers (1983) suggested that mezerein may act on a subset of the

phorbol ester receptor, perhaps a down-modulated form of PKC. However, following the identification of the PKC isoforms, the actions of mezerein described in this study seem likely to be explained by selective effects of this agent at certain isoforms. Certainly, mezerein shows selective binding to some of the multiple phorbol ester binding sites in mouse skin (Dunn and Blumberg, 1983) which may represent one or more PKC isoform. Short chain deoxyphorbols, e.g. 12-deoxyphorbol 13-isobutyrate and some phorbols with unsaturated side chains, e.g. phorbol 12-retinoate 13-acetate may also be able to discriminate subpopulations of phorbol ester recognition sites (Dunn and Blumberg, 1983). However, it is possible that these agents can act at least in part, on other (as of yet unidentified) kinases, which may be closely related to PKC.

Purified PKC subspecies do show heterogeneity in their responsiveness to different phorbol esters and phorbol ester-related compounds. Evans and his co-workers (1991) showed that the β isoform could be selectively activated by 12-deoxyphorbol 13-phenylacetate 20-acetate (DOPPA) over the α, γ and δ isoforms. However, in this study, the effect of DOPPA on ε, ζ, η, λ and θ isoform activity was not examined and it was admitted that the β isoform preparation may be contaminated by B series isoforms. Further investigations into the effect of DOPPA on the activity of purified isoforms, prepared, for example, using the baculovirus-insect cell expression system, will clarify any isoform-selective nature of DOPPA. Other phorbol ester analogues, such as thymeleatoxin and sapintoxin-A are also reported to show selectivity towards certain isoforms. Thymeleatoxin (a close congener of mezerein) is a relatively poor activator of the δ and ε PKC isoforms, but can activate the A series PKCs (although in a manner which is highly dependent upon Ca^{2+}), and sapintoxin-A is a potent activator of the α, β and γ forms, but fails to activate the δ and ε isoforms effectively (Evans *et al,* 1991; Ryves *et al,* 1991).

Further development of selective activators of different PKC isoforms will be useful for future studies into the of involvement of these enzymes in specific cellular responses.

1.2.4.2 Protein kinase C inhibitors

Protein kinase C inhibitors have been widely used to study the involvement of this enzyme in responses to various agonists. These agents may inhibit PKC activity by interfering with the Ca^{2+}/phospholipid interaction, phorbol ester/DAG binding or ATP or substrate binding.

Inhibitors that prevent the interaction of PKC with Ca^{2+} include, dibucaine, adriamycin, polymyxin B and melittin (Huang 1989). However, since these agents block the activity of other Ca^{2+}-dependent enzymes, they are rarely used as PKC inhibitors. Other agents that interact with the PKC regulatory domain include calphostin C and sphingosine. Calphostin C inhibits PKC activity by interfering with DAG/phorbol binding and has reasonable selectivity for PKC over other protein kinases (Kobayashi *et al,* 1989). However, calphostin C seems to cause permanent inactivation of phorbol ester binding and this may limit its usefulness in some studies. Sphingosine is an 18-carbon chain lipid base which potently inhibits PKC activity by interacting competitively with DAG/Ca^{2+} and phosphatidylserine binding (Hannun and Bell, 1987), but also has effects on other biological processes which are independent of its ability to inhibit PKC (Hidaka and Kobayashi, 1992). Potent peptide inhibitors of PKC activity have been developed which resemble the pseudosubstrate region of PKC (House and Kemp, 1987; Eichholtz *et al,* 1990). Since this region varies between different isoforms, it may be possible to develop isoform-selective peptide inhibitors which act at this site. However, these inhibitors are not cell permeable, limiting their usefulness.

Compounds which inhibit PKC activity by interacting with the catalytic domain include the isoquinolinesulphonamides, such as 1-(5-isoquinolinesulphonyl)-

2- methyl piperazine (H7), and compounds which contain the indole carbazole moiety, e.g. staurosporine, K252a. Kinetic studies have revealed that the action of H7 on PKC activity is reversible and competitive with ATP. However, since the ATP binding sites of a number of serine/threonine kinases show considerable sequence homology, it is not surprising that H7 only shows modest selectivity for PKC over other protein kinases. For example, H7 can inhibit PKC, cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) activities all with Ki values in a range of 3 - 6 μM (Hidaka and Kobayashi, 1992). Since the H7 analogue, HA 1004, has similar inhibitory effects on a number of serine/threonine-specific protein kinases, but has reduced affinity for PKC, this compound is a useful control for determining the actions of PKC (Hidaka *et al,* 1984). Staurosporine and K252a also appear to inhibit PKC activity by interacting with the catalytic domain, perhaps by competing with the ATP binding site (Kase *et al,* 1986; Nakadate *et al,* 1988; Huang, 1989). However, both compounds inhibit the activity of other kinases by similar mechanisms and cannot be considered as a selective inhibitors of PKC (Rüegg and Burgess, 1989). Davis *et al* (1989) have developed more selective analogues of staurosporine. One such bis-indolyl maleimide compound, Ro 31-8220, has 100 fold greater selectivity than staurosporine for PKC over PKA. More recently, further development of these compounds has produced a series of new drugs with even greater selectivity for PKC. One such compound, Ro 31-8425, is 350 fold more selective for PKC than PKA (Nixon *et al,* 1992), but has yet to become commercially available. A natural product, chelerythrine, is 250 fold more potent at inhibiting PKC than PKA (Herbert *et al,* 1990a), and has very recently become commercially available.

Although PKC inhibitors which interact with the catalytic domain are generally regarded to be unable to distinguish between different PKC isoforms, H7, in particular, can selectively inhibit some cellular actions of phorbol esters, but not others. In mouse epidermal cells, H7 inhibited PMA-stimulated ornithine

decarboxylase induction, but not the PMA-induced decrease in epidermal growth factor binding. Sphingosine, on the other hand, could inhibit both PMA-mediated responses (Nakadate *et al,* 1989). The phorbol ester-stimulated activity of a protein kinase purified from porcine spleen was less sensitive to block by K252a than PKCs purified from mouse brain (Gschwendt *et al,* 1989). This K252a-resistant PKC may be a form of the δ isoform (Uchida *et al,* 1991). However, these PKC-inhibitor resistant actions of phorbol esters may represent effects on processes which are independent of PKC involvement. Alternatively, PKM has been reported by some to show reduced potency for H7 and staurosporine (Junco *et al,* 1990), although Nakadate *et al* (1988) reported that such potency shifts did not occur. The phenomenon observed by Junco and co-workers may therefore be due to further, deleterious proteolysis of the catalytic subunit. Alternatively, PKM originating from different PKC isoforms may have different pharmacological properties. Nevertheless, the possibility that PKC isoform-specific inhibitors may exist is an interesting prospect. Selective inhibitors of certain PKC isoforms will provide a greater understanding regarding isoform-specific functions and may have novel therapeutic actions since PKC is implicated in pathological states such as cancer, inflammation, and neuronal damage (Nishizuka, 1988).

1.3. RECEPTOR-CONTROLLED ARACHIDONIC ACID RELEASE

Phospholipids (and certain species of PtdCho and phosphoinositides in particular) possess stearate on the 1 carbon and arachidonate on the 2 carbon of the *sn*-1,2-DAG backbone (Billah and Anthes, 1990; Meldrum *et al,* 1991). Agonist-stimulation of a number of receptor types can enhance arachidonic acid (AA) liberation from these membrane phospholipids (Irvine 1982); the action of PLA_2 is implicated as an important route of AA release. Phospholipase A_2 catalyses the hydrolysis of the 2-position of the phospholipid, liberating equimolar amounts of free fatty acid and lysophospholipid (Irvine, 1982) (Figures 1.1 and 1.3) and since the

majority of AA in mammalian cells is esterfied in the 2-acyl position of certain phospholipids (Irvine, 1982), this will have the effect of increasing free AA levels. Alternatively, PLC- or PLD-mediated phosphoinositide or phosphatidylcholine breakdown may produce arachidonyl-diglycerides (Figure 1.3). The AA present in these DAGs can be liberated either by the action of DAG lipase (Bell *et al,* 1979; Irvine, 1982) at the 2-position, or by the sequential action of DAG-lipase at the 1-position, followed by monoacylglycerol lipase (MAG lipase) action on the 2-monoacylglycerol (Prescott and Majerus, 1983) (Figure 1.3).

Arachidonic acid has a range of biological effects (see section 1.3.5), and can be metabolised further to prostaglandin, leukotriene and epoxygenase products which also have biological activity (Needleman *et al,* 1986). Subsequently, the receptor control of AA release has been suggested to be functionally important.

1.3.1 The phospholipase A_2 enzyme family

A variety of extracellular and intracellular forms of PLA_2 have been described. The secreted PLA_2 forms are of low molecular weight (12 - 18 kDa) and function in phospholipid digestion and inflammatory responses, whereas the high molecular weight (30 - 110 kDa) PLA_2s are cytosolic and are probably involved in receptor-linked signal transduction mechanisms.

In the gastro-intestinal tract, PLA_2 action is required for phospholipid breakdown and digestion, for example, in the pancreas, where PLA_2 is secreted as an inactive zymogen which is converted to the active form by proteases (Chang *et al,* 1987a). The protein structures of the pancreatic PLA_2 enzymes have been well characterised and are related to the PLA_2 enzymes found in snake venom, bee venom (Verheij *et al,* 1981) and rat stomach (Yasuda *et al,* 1990). In aqueous solution, these PLA_2s exist as dimers and are rich in disulphide bonds which are essential for their stability and activity (Verheij *et al,* 1981). These secreted forms of PLA_2 are

dependent upon millimolar levels of Ca^{2+} for full activity (Ono *et al,* 1988) and have a putative Ca^{2+} binding domain (van Sharrenburg *et al,* 1985).

High levels of PLA_2s are found in arthritic joints (Vadas *et al,* 1985) and when purified PLA_2 protein is injected intradermally, it can cause an acute inflammatory reaction (Murakami *et al,* 1990), suggesting that PLA_2 has a role in inflammatory disease. Phospholipase A_2 has also been implicated in the pathogenesis of asthma, where it hydrolyses PtdCho in the lung surfactant, and may be the cause of acute respiratory distress syndrome (Heath *et al,* 1985). The inflammatory PLA_2 forms are of low molecular weight but have different primary structures from the digestive and snake venom enzyme (Henrikson *et al,* 1977). Nevertheless, the inflammatory and pancreatic PLA_2s show close similarity in their Ca^{2+}-binding regions (Wery *et al,* 1991) and are also dependent upon millimolar Ca^{2+} levels for activation (Vadas *et al,* 1985). Related forms are found in liver (Aarsman *et al,* 1989), rat spleen (Ono *et al,* 1988) and in rat platelets (Hayakawa *et al,* 1988).

Cytosolic, high molecular weight PLA_2s have been described in various different cell types including rat kidney (Gronich *et al,* 1990) and sheep platelets (Loeb and Gross, 1986). Unlike the low molecular weight forms, the high molecular weight forms are active over a range of concentrations of Ca^{2+} (300 - 800 nM) which are equivalent to those in agonist-stimulated cells. The cytosolic PLA_2 forms in the human monocytic U937 cell line are amongst those best characterised where a PLA_2 with a molecular mass of approximately 100 kDa has been identified (Clark *et al,* 1990, 1991a). This PLA_2 prefers substrates with AA at the 2-position and is activated by Ca^{2+} at levels equivalent to those found in stimulated cells, which led to the suggestion that this PLA_2 is responsible for agonist-induced eicosanoid production in U937 cells. Molecular cloning and expression studies have revealed that this PLA_2 has no detectable sequence homology with the characterised secretory PLA_2 forms. Most interestingly this PLA_2 has an N-terminal 140 amino acid fragment which translocates to natural membrane vesicles in a Ca^{2+}-dependent fashion. This portion

of the enzyme has sequence homology with the Ca^{2+}-binding domain of PKC-γ, to the synaptic vesicle protein p65 (which has been implicated in vesicle fusion with the plasmalemma), with the GTPase activating protein and with PLC-γ. Such a domain may have an important regulatory role and may control the association of PLA_2 with the membrane substrate, in a Ca^{2+}-dependent manner, during a stimulus.

The various high molecular weight PLA_2s have distinct specificities for different phospholipid substrates. A cytosolic PLA_2, partially purified from RAW 264.7 macrophage cells, preferentially hydrolyses PtdCho (Leslie *et al,* 1988), whilst a form in rat kidney hydrolyses PtdCho and phosphatidylethanolamine equally well (Gronich *et al,* 1990). In platelets, a PLA_2 form has been reported to specifically hydrolyse PtdOH (Lapetina *et al,* 1981). The physiological significance of the substrate preference of these different PLA_2 forms has yet to be determined, although such actions would generate different species of lysophospholipid which may, in principle, have varying degrees of biological activity. Nevertheless, these cytosolic PLA_2s, in general, selectively hydrolyse substrate with AA at the *sn*-2 position (Kim *et al,* 1988; Leslie *et al,* 1988; Clark *et al,* 1990; Channon and Leslie, 1990; Dietz and Mong, 1990) and their actions would be expected to influence free AA levels, irrespective of the species of lysophospholipid produced.

1.3.2 Hormonal regulation of phospholipase A_2 activity

The low molecular weight forms of PLA_2 are unlikely to be directly controlled by receptor activation since they require Ca^{2+} levels which are higher than those found within a cell following receptor activation. Furthermore, these enzymes are located within secretory granules and are unlikely to be affected by changes in the intracellular environment. In any case, the reducing environment of the cytosol would inactivate the disulphide rich secreted PLA_2 forms. In contrast, the high molecular weight cytosolic PLA_2 forms are resistant to the reducing environment of the cytosol, are active at agonist-induced levels of Ca^{2+} and are the likely candidates

for the receptor controlled enzyme. It has been suggested that the cytosolic PLA_2 forms may be activated by second messengers generated by the PtdIns(4,5)P_2-PLC pathway or may be directly activated via specific G-proteins.

1.3.2.1 Role of calcium in phospholipase A_2 activation

In certain cell types, PLA_2 activation was found to be closely linked to the occupancy of Ca^{2+} mobilising receptors. In human platelets, thrombin-induced AA release was reduced in presence of the Ca^{2+} chelator, quin 2, and in the absence of extracellular Ca^{2+} (Simon *et al,* 1986), suggesting that PLA_2 activity is controlled by changes in intracellular Ca^{2+} levels. These studies supported the hypothesis that receptor activation of PLA_2 may be secondary to PLC-catalysed inositol phospholipid hydrolysis and Ca^{2+} mobilisation. However, in a number of different cell types, including HL60 cells, an increase in intracellular Ca^{2+} levels alone was found to be insufficient to activate PLA_2 (Billah *et al,* 1986). Furthermore, the mechanism of receptor-induced AA release can be dissociated from inositol phospholipid turnover in FRTL5 rat thyroid cells (Burch *et al,* 1986, see also section 1.3.2.3), spinal cord neurons (Kanterman *et al,* 1990) and Madin-Darby canine kidney cells (Slivka and Insel, 1987), indicating that factors other than or in addition to changes in intracellular Ca^{2+} levels may control PLA_2 activity.

1.3.2.2 Role of protein kinase C in phospholipase A_2 activation

Several studies show that PKC can have an important regulatory role in the control of PLA_2 activity by certain agonists. In cultured astrocytes, the PKC activator PMA can elicit prostaglandin E (PGE) release, supporting a role for PKC in the control of PLA_2 activity (Hartung and Toyka, 1987). Furthermore, in Madin-Darby canine kidney cells, PKC down regulation reduces bradykinin-induced AA release suggesting that PKC may modulate PLA_2 activity in these cells (Godson *et al,* 1990). More direct evidence for PKC control of PLA_2 activity came from studies which showed that PMA treatment of intact rat renal mesangial cells caused an

increase in PLA_2 activity measured in cell-free extracts (Gronich *et al,* 1988). Interestingly, treatment with PMA alone could not mimic the level of AA release measured from intact mesangial cells stimulated with the physiological agonist, arginine vasopressin (AVP) (Gronich *et al,* 1988). However, in intact cells, PMA could facilitate Ca^{2+} ionophore-induced PLA_2 activity, as assessed by measuring PGE_2 release, to levels that mimicked those obtained by stimulation with AVP (Bonventre and Swidler, 1988). Therefore, both PKC activation and Ca^{2+} may act synergistically to activate PLA_2 in these cells. Alternatively, raised intracellular Ca^{2+} may promote the activation of some PKCs by PMA although it is not clear whether this contributes to the synergy observed in these studies.

Protein kinase C has been proposed to modulate PLA_2 activity by a number of different mechanisms. For example, PKC may activate PLA_2 by phosphorylating the enzyme or by altering the activity of inhibitory or stimulatory influences on PLA_2 action. Purified PKC-β can phosphorylate a cytosolic PLA_2 purified from macrophage cells *in vitro,* but without any effect on the catalytic activity of PLA_2 on phospholipids from macrophage membranes (Wiljkander and Sunder, 1991). It has yet to be determined if direct phosphorylation of PLA_2 by other PKC isoforms can occur in intact cells and whether this is related to PLA_2 activation.

The lipocortins are putative endogenous inhibitors of PLA_2 activity whose actions are suggested to be modulated by PKC phosphorylation. In platelets, both thrombin and PMA increase the phosphorylation state of a lipocortin-related protein and this was suggested to decreases its anti-PLA_2 activity (Tourqui *et al,* 1986). Protein kinase C phosphorylation and inactivation of lipocortin would be a suitable mechanism by which PLA_2 activity could be controlled. However, a study by Crouch and Lapetina (1986) showed that, although both α and γ-thrombin induced phosphorylation of this protein, only α-thrombin could induce AA release. Thus, there was a clear dissociation between the ability of an agonist to phosphorylate this protein and release AA. Furthermore, in cell-free assays, the actions of lipocortin can

be overcome by high phospholipid substrate concentrations (Davidson *et al,* 1987), indicating that lipocortins do not interact directly with PLA_2, as was first suggested, but exert their action by binding to the phospholipid substrate. Such actions may account for the ability of these agents to inhibit purified PLC and PLD activity *in vitro* (Hirata, 1981). These observations question the specificity of lipocortins and their significance as regulators of cytosolic PLA_2 activity.

Recently, protein kinase C has been suggested to modulate the activity of a phospholipase A_2-activating protein (PLAP) which has functional and antigenic properties related to the PLA_2 stimulatory peptide, melittin (Clark *et al,* 1987a; Crooke *et al,* 1989). In the leukotriene D_4 receptor signal transduction system, PKC regulates the transcription, translation and synthesis of a PLAP which can activate a PtdCho-specific PLA_2, but has no effect on PLC activity or on pancreatic or snake venom PLA_2 (Clark *et al,* 1987a; 1991b). Thus, PLAP may be a regulator of cytosolic PLA_2 activation in response to an agonist.

In other cell types both PKC-dependent and -independent pathways of AA release have been described. For example, in endothelial cells, bradykinin-induced AA release was not reduced by the PKC inhibitor, staurosporine, whereas PMA-induced AA release was, suggesting that bradykinin activates PLA_2 in a PKC-independent manner. Furthermore, bradykinin- and PMA-induced AA release were additive suggesting that each stimulus induces AA release by a distinct mechanism (Buckley *et al,* 1991).

Diacylglycerols may activate PLA_2 by a PKC-independent mechanism. Partially purified human platelet PLA_2 activity was stimulated by DAG in cell-free assay (Kramer *et al,* 1987; Burch, 1988). Although PKC contamination was present in the PLA_2 preparation, the PKC inhibitor, H7 had no effect on DAG-induced PLA_2 activation (Burch, 1988), suggesting that DAG may activate PLA_2 independently of PKC involvement. Diacylglycerol may activate PLA_2 by having a physico-chemical action on the structure and stability of the phospholipid bilayer which may enhance

the access of the protein to the substrate. Alternatively, DAG may induce conformational changes in PLA_2 itself. However, any possible physiological significance of this action of DAG has yet to be verified.

1.3.2.3 G-protein control of phospholipase A_2 activity

The possible coupling of PLA_2 to receptors via G-proteins was initially indicated by studies from Burch and his co-workers (1986). Prior treatment of intact FRTL 5 cells with pertussis toxin inhibited noradrenaline- and GTP-analogue-induced AA release, suggesting that α_1-adrenergic receptors in these cells are to coupled to PLA_2 via a G-protein. However, GTP analogues are potent activators of all G-protein dependent processes, including PLC activation, and since many receptors couple to both PLC and PLA_2, this could indicate that the second messenger products of PLC action can stimulate PLA_2 activity (see sections 1.3.2.1 and 1.3.2.2). As a consequence, further evidence was sought to support the coupling of receptors to PLA_2 via G-proteins. Additional experiments showed that; (1) pertussis toxin, which ADP ribosylates and inactivates certain G-protein α subunits, selectively inhibited noradrenaline- and GTP-γ-S-induced AA release but not inositol phosphate accumulation; (2) the PLC inhibitor, neomycin, blocked GTP-γ-S-induced inositol phosphate formation but not AA release; and (3) that AA release could be induced from [^{3}H]-arachidonoyl PtdCho by GTP-γ-S using FRTL 5 cell membranes (Burch *et al,* 1986). These results suggested that the α_1-adrenergic receptor in FRTL 5 cells is associated with 2 G-proteins; a pertussis toxin-sensitive form which activates PLA_2 and a pertussis toxin-insensitive form which controls PLC activity. Although activation of PLA_2 and PLC may be controlled by distinct G-proteins, it is clear that the G-protein control of PLA_2 activity in some systems may still depend upon PLC action to some extent. In neutrophils, fMet-Leu-Phe activation of PLA_2 is probably under dual control from a Ca^{2+}/PKC-dependent pathway and a G-protein dependent pathway (Cockcroft *et al,* 1991) with approximately 50% of AA arising via a G-

protein-dependent route. However, micromolar Ca^{2+} levels are required for G-protein-dependent AA release, indicating that this pathway may still require prior activation of PLC.

Further studies on the G-protein control of PLA_2 activity have been carried out using rod outer segment of the bovine retina. In this system, the light receptor for the rod outer segments couples to both PLA_2 and cyclic GMP-dependent phosphodiesterase by a G-protein known as transducin (Jelsema, 1987). When purified α and $\beta\gamma$ transducin subunits were added to membranes, the α subunit stimulated the cyclic GMP-dependent phosphodiesterase. Surprisingly, PLA_2 activity was stimulated by the $\beta\gamma$ complex, but not by the α subunit (Jelsema and Axelrod, 1987). Thus, the $\beta\gamma$ subunit complex was suggested to modulate PLA_2 activity. Similarly, patch clamp studies have show that purified $\beta\gamma$ subunits open cardiac plasma membrane K^+ channels apparently by stimulating PLA_2 activity (Kim *et al,* 1989). However, although the $\beta\gamma$/PLA_2 response in heart was attenuated by the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), the actions of the physiological regulator of these channels, acetylcholine, was unaffected by this drug. These results question the role of the $\beta\gamma$ subunits and PLA_2 activation in the physiological control of these channels. Furthermore, the concentration of $\beta\gamma$ subunits that activated these K^+ channels was over 200 fold higher than the concentration of α_i subunit reported by another group to elicit the same effect (Codina *et al,* 1987; Logothetis *et al,* 1987). This evidence supports a role for the α but not the $\beta\gamma$ subunits in the agonist-control of heart K^+ channel opening. Thus, the α subunit is a more likely candidate as the main G-protein effector of agonist-induced PLA_2 activation although the $\beta\gamma$ subunits, at higher concentrations, may also be effective.

Attempts to identify an α subunit type which activates PLA_2 show that both pertussis toxin and cholera toxin (which causes sustained activation of $G_s\alpha$) can inhibit and stimulate PLA_2 activity (Jelsema, 1987), suggesting that multiple forms of

G-protein may couple to PLA_2. In one study, Gupta *et al* (1990) found that, in Chinese hamster ovary (CHO) cells, thrombin and type 2-purinergic receptor agonists stimulated PLA_2 activity through a G_i-type G-protein. In an attempt to identify the functional domain of the α subunit, chimeric cDNAs were constructed, with different lengths of $G_s\alpha$ subunit cDNA being replaced with corresponding bases from $G_i\alpha$, and the chimeric constructs were expressed in CHO cells. These experiments showed that the last 36 amino acids of $G_i\alpha$ were critical for G-protein regulation of PLA_2 activity. This type of approach has the advantage over pharmacological studies in that one can examine the involvement of certain G-protein α subunits in receptor-effector coupling with greater selectivity. Such studies, together with reconstitution experiments using specific G-protein α subunits, receptors and effector enzymes, may clarify the nature of the G-proteins which couple to PLA_2. Microinjection of H-*ras* (which encodes a 21 kDa monomeric G-protein) into fibroblasts can activate PLA_2, without having an effect on inositol phospholipid metabolism (Bar-Sagi and Feramisco, 1986), indicating that monomeric G-proteins may also control PLA_2 activity.

1.3.3. Pharmacological regulation of phospholipase A_2 activity

Since PLA_2 activation has an important role in receptor signalling and inflammatory disease, selective PLA_2 inhibitors may be effective therapeutic agents. Inhibitors of PLA_2 can affect enzyme activity by a number of different mechanisms; by either interacting with the enzyme itself, or by interfering with the enzyme/substrate interaction or by altering Ca^{2+} availability.

Compounds which make the membrane more fluid (e.g. halothane) or rigid (cholesterol) disturb the architecture of the lipid-water interface, and in doing so, may physically prevent PLA_2 action. Other drugs, such as local anaesthetics, polyamines, antipsychotic agents, antibiotics, organic solvents, and antimalarial agents interact with the phospholipid substrate and prevent enzyme interaction (Chang *et al*, 1987a). The antimalarial drugs chloroquine and quinacrine (mepacrine)

are amongst those which have been most widely used to inhibit PLA_2 activity. In addition to their actions on the phospholipid substrate, these agents may also inhibit enzyme activity by altering Ca^{2+} availability (Chang *et al,* 1987a). Clearly, a number of cellular processes require Ca^{2+} and any agent which alters Ca^{2+} availability will not be selective. Such actions may account for the ability of quinacrine to inhibit platelet PLC activity, although at concentrations that are 10 times greater than those which are required to block PLA_2 activity (Hofmann, 1982).

The lipoxygenase and cyclo-oxygenase inhibitors, indomethacin, 5,8,11,14-eicosatetraynoic acid (ETYA) and NDGA can inhibit PLA_2 activity from neutrophil extracts although at concentrations 2 or more fold greater than those needed to inhibit the relevant pathways of AA metabolism (Franson *et al,* 1980; Lanni and Becker, 1985). Since agonist-induced eicosanoid production is dependent upon both PLA_2 and lipoxygenase/cyclo-oxygenase activity, it is important to interpret the actions of these drugs with due care.

Various agents can inhibit PLA_2 activity by directly interacting with the enzyme. ρ-Bromophenacyl bromide inhibits PLA_2 activity by covalently modifying essential histidine residues which are associated with the catalytic site of the enzyme (Volwerk *et al,* 1974; Drenth *et al,* 1976; Roberts *et al,* 1977). However, this drug can also modify a variety of other proteins and can inhibit platelet PLC activity by modifying sulfhydryl groups (Hofmann *et al,* 1982; Kyger and Franson, 1984). Manoalide covalently modifies lysine residues near the PLA_2 active site, inhibiting enzyme activity, but can inhibit the actions of other phospholipases by similar mechanisms (Lombardo and Dennis, 1985).

A number of alternative PLA_2 inhibitors have been recently developed. The structure of the Roche PLA_2 inhibitors, Ro 31-4493 and Ro 31-4639, has been based on the X-ray crystal structure of the porcine PLA_2 active site (Davis *et al,* 1988) and can inhibit PLA_2 activity in ram sperm sonicates (Roldan and Mollendo, 1991). These compounds appear to block activity by interacting either directly or

allosterically with the enzyme active site and, therefore, may have a degree of specificity. Other new PLA_2 inhibitors, such as ONO-RS-082 and aristolochic acid, can block PLA_2 activity without altering PLC activity (Banga *et al,* 1986; Rosenthal *et al,* 1989). The exact mechanism of action of these two agents has still to be determined, although aristolochic acid can interact directly, but non-covalently with isolated PLA_2. Such agents may prove useful for examining the involvement of PLA_2 in physiological responses.

The bee venom peptide, melittin can activate PLA_2 (Habermann, 1972) without altering PLC and lysophospholipase activity (Shier, 1979). The exact mechanism by which melittin activates PLA_2 is unknown, although melittin can intercalate into cell membranes (Mix *et al,* 1984) which may render the phospholipid substrate more susceptible to PLA_2 hydrolysis. Melittin can also increase intracellular Ca^{2+} levels, possibly by forming ion-permeable 'channels' (Tosteson and Tosteson, 1981), and this action of melittin may also, in part, contribute to activation of PLA_2. However, at high concentrations, melittin can lyse cells by disrupting cell membrane integrity (Mix *et al,* 1984). These actions of melittin may account for its non-specific effects (Metz, 1986).

1.3.4 Alternative pathways of arachidonic acid release

In platelets, thrombin can induce a rapid release of radioactive AA from pre-labelled cells. However, when PLA_2 activity was measured in platelet extracts, this activity was too low to account for the rapid release of AA observed with thrombin (Bell *et al,* 1979). This observation led to the hypothesis that AA may be released from phospholipids by a route other than that involving PLA_2. Further experiments showed that upon thrombin stimulation of platelets, the levels of 1-stearoyl-2-arachidonoyl containing phospholipids, but not other types of phospholipid, were decreased (Broekman *et al,* 1979) and that a rapid increase in 1-stearoyl-2-arachidonoyl diglyceride occurred (Bell *et al,* 1979). Arachidonate was

released from this diglyceride by the action of diglyceride lipase in the particulate fraction of platelet sonicates (Bell *et al,* 1979). Thus, the sequential actions of an inositol phospholipid-specific PLC, liberating arachidonyl diglyceride, followed by the action of a DAG lipase (Figure 1.3) may be an important route of agonist-induced AA release in some cell types. Since certain species of PtdCho are enriched in AA at the *sn*-2 position (Billah and Anthes, 1990), it is of course, possible that arachidonyl-containing diglycerides can be produced by the actions of either a PtdCho-specific PLC or by the action of PLD, followed by PPH (section 1.1.2) and that DAG lipase action can generate AA from this DAG. However, the physiological significance of the PtdCho-PLC/DAG lipase and PLD/DAG lipase pathways of AA release has still to be determined.

More recent studies have shown that 1,2-DAG, produced in response to thrombin, was preferentially hydrolysed at the *sn*-1 position over the *sn*-2 position, since a 2-monoacylglycerol intermediate, but no 1-monoacylglycerol was detected (Prescott and Majerus, 1983). Thus, in this model, it would seem that DAG lipase will catalyse the removal of stearate at the 1-position. Arachidonate will then be cleaved from the 2-monoacylglycerol by MAG lipase action (Figure 1.3). The MAG lipase activity can be separated from the DAG lipase activity on DEAE-Sepharose (Prescott and Majerus, 1983), suggesting that these activities may represent distinct enzymes.

In the F-11 dorsal root ganglia x neuroblastoma hybrid cell line, bradykinin could induce a significant increase in [^{3}H]-AA release from prelabelled cells (Morell *et al,* 1991). Since bradykinin was unable to induce the appearance of lysophospholipid, it was suggested that PLA_2 was not involved in the mechanism of AA release by this agonist. However, it is now clear that some high molecular weight cytosolic forms of PLA_2 exhibit lysophospholipase activity (Leslie, 1991). Thus, the lack of appearance of lysophospholipid in agonist-stimulated cells may not indicate conclusively the involvement of PLA_2 in the mechanism of AA release. The

involvement of either the PLA_2 or DAG lipase pathways in agonist-induced AA release may be further assessed using PLA_2 inhibitors (section 1.3.3) and the DAG lipase inhibitor, 1,6-di(*O*-(carbamoyl)cyclohexaneoxime)hexane (RHC 80267). For example, luteinizing hormone-releasing hormone (LHRH) induced luteinizing hormone (LH) release from rat anterior pituitary cells was reduced by RHC 80267, at a concentration known to selectively inhibit DAG lipase action (10 μM), to levels approximately 60% of control. The remaining response to LHRH was blocked by the PLA_2 inhibitor, quinacrine (Chang *et al,* 1988), suggesting that the actions of both PLA_2 and DAG lipase may be important for secretory responses to LHRH. However, RHC 80267 clearly lacks specificity at higher concentrations and can inhibit the activity of PLA_2, PLC and cyclo-oxygenase at concentrations of 100 μM and above (Oglesby and Gorman, 1984). As a consequence, care must be taken when interpreting the effect of this agent on cellular responses.

Arachidonic acid release may also be increased by inhibiting the activity of the enzymes involved in the re-esterification of AA. Phorbol esters and diglycerides can increase AA release from platelets by a mechanism which involves inhibition of the activity of arachidonoyl CoA synthetase and arachidonoyl lysophosphatidate acyltransferase (Fuse *et al,* 1989). Although the exact mechanism by which this inhibition of enzyme activity occurs is unknown, PKC may have either direct or indirect actions on enzyme activity. Any inhibitory action of PKC on arachidonoyl CoA synthetase and arachidonoyl lysophosphatidate acyltransferase activities would provide another hypothetical mechanism by which an agonist could induce an increase in free AA levels.

1.3.5 Cellular actions of arachidonic acid and arachidonic acid metabolites

Arachidonic acid has a putative role as an intracellular messenger in mammalian cells and can be metabolised further, leading to the formation of biologically active eicosanoids; cyclo-oxygenase and associated enzymes form both

prostaglandins (PGs) and thromboxanes (TXs); lipoxygenases form leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs); and the cytochrome P-450 epoxygenases generate various epoxide metabolites (Figure 1.3) (see Needleman *et al,* 1986 for review).

In vitro, AA and its metabolites can have many different cellular actions which may contribute to their physiological effects. For example, AA can stimulate guanylate cyclase activity (Gerzer *et al,* 1986), adenylate cyclase activity (Poon *et al,* 1981) and can increase inositol phospholipid turnover by activating PLC (Irvine *et al,* 1979). Thus, AA can have positive actions on a number of different second messenger pathways which may serve to stimulate or amplify them, possibly having important functional consequences. For example, in placental cells, AA induces human placental lactogen secretion, probably by stimulating PLC activity and inositol phospholipid hydrolysis in these cells (Zeitler and Handwerger, 1985). Since AA and certain AA metabolites can induce Ca^{2+} release from intracellular Ca^{2+} pools, including those which are sensitive to $Ins(1,4,5)P_3$ (Beaumier *et al,* 1987), and PLC may be a Ca^{2+}-stimulated enzyme (Meldrum *et al,* 1991), a Ca^{2+} mobilising action of AA could alter PLC activity. Arachidonic acid can also induce prolactin secretion from pituitary GH_3 cells by a mechanism which is not prevented by inhibitors of lipoxygenase or cyclo-oxygenase pathways (Kolesnick *et al,* 1984). In contrast, using dispersed anterior pituitary cells, the inhibitors of AA metabolism, ETYA and NDGA, can inhibit LHRH-induced LH release (Hulting *et al,* 1984, 1985) suggesting that AA metabolites are involved in this process.

Arachidonic acid, itself, and some of its metabolites, notably 12-(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) and 5(S),6(R),15(S)-trihydroxy-7,9,13-trans,11-cis eicosatetraenoic acid (Lipoxin A4), can activate the γ (and to a lesser extent, the α and β isoforms) of PKC (Naor *et al,* 1988; Shearman, 1989a, section 1.2.2). In platelets, AA can induce phosphorylation of certain endogenous substrates; a response which can be prevented by the PKC inhibitor, H7,

and by immunodepletion of PKC, but which is mimicked by phorbol esters, suggesting that AA induces this response by modulating PKC activity (Khan *et al,* 1991). Furthermore, AA may induce platelet aggregation by directly stimulating PKC activity (Nishikawa *et al,* 1988).

Arachidonic acid and its metabolites may also have an important role in the control of neurotransmitter release. Electrophysiological experiments have shown that AA and the lipoxygenase metabolite, 12-HPETE, increased the opening probability of a subclass of K^+ channels in *Aplysia* that are suggested to participate in presynaptic inhibition of neurotransmitter release by Phe-Met-Arg-Phe-NH_2 (FMRFamide) (Piomelli and Greengard, 1990). Furthermore, AA and 12-HPETE could inhibit the activity of Ca^{2+}/calmodulin-dependent protein kinase II (Piomelli *et al,* 1989), an enzyme whose actions are important for synaptic vesicle release. Thus, the combined action of 12-HPETE on *Aplysia* K^+ channel activity and Ca^{2+}/calmodulin-dependent protein kinase II activity have been suggested to inhibit neurotransmitter release (Piomelli and Greengard, 1990). Arachidonic acid and LTC_4 can also directly activate K^+ channels in atrial myocytes (Kurachi *et al,* 1989) which would presumably cause cell hyperpolarization and thereby inhibit smooth muscle excitation.

Arachidonic acid and its metabolites can diffuse out of the cell and act, either on the cell from which they are released, or on adjacent cells. By having such an action, AA has been proposed as a retrograde messenger for long-term potentiation (LTP) in the hippocampus. Experiments by Williams and collaborators (1989) show that following N-methyl D-aspartate-activation of the dentate gyrus, AA is released from post-synaptic cells. Perifusion with AA, together with a weak stimulation of the fibres connecting the entorhinal cortex to the dentate gyrus of the hippocampus, caused a long-term enhancement of synaptic transmission in the dentate gyrus of the anaesthetised rat, similar to LTP. However, the cellular site and mode of action of AA in the mechanism of LTP is uncertain.

Several membrane associated receptors for prostaglandins, thromboxanes and leukotrienes are now well established, underlying the importance of these eicosanoids in cell to cell signalling. Leukotrienes have a number of biological actions, many of which may be mediated by their interactions with specific receptors. For example, LTB_4 and LTC_4 can cause constriction of the bronchi, arterioles, dilation of the venules and can cause arrhythmia (Needleman *et al,* 1986). Leukotriene B_4 is also a potent chemotactic agent for neutrophils and eosinophils (Needlemann *et al,* 1986). Thus, the leukotrienes have been implicated as mediators of asthma, inflammatory reactions and myocardial infarction. Prostaglandins can also act at specific receptors, causing a number of cellular effects. For example, PGD_2 can induce smooth muscle contraction, sleep induction, hypothermia, transmitter release, antiaggregation of blood platelets and growth inhibition (Shimizu and Wolf, 1990). Thus, AA metabolites have a wide range of cellular actions which can be mediated by their interaction with specific membrane bound receptors.

1.4 THE MOLECULAR MECHANISMS OF LUTEINIZING HORMONE-RELEASING HORMONE ACTION IN THE ANTERIOR PITUITARY

The anterior pituitary gland consists of at least 5 different cell types which release different hormones; the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are released from gonadotrophs; prolactin is released from lactotrophs; growth hormone (GH) is released from somatotrophs; adrenocorticotrophic hormone is released from corticotrophs and thyroid-stimulating hormone (TRH) is released from thyrotrophs. The release of these hormones is under the control of a number of factors, many of which are released from the nerve endings of the median eminence of the hypothalamus, and are carried to the pituitary gland by the hypophysial-portal blood vessels. Hypothalamo-pituitary function is important for controlling physiological processes such as growth, stress, water retention, fertility and metabolic rate (Fink, 1988).

The hypothalamic decapeptide, luteinizing hormone releasing hormone (LHRH, pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) has four main actions on the anterior pituitary gland; (1) it can induce LH and FSH release; (2) it can increase gonadotroph responsiveness to itself - an effect known as LHRH priming (Aiyer *et al,* 1974a; Edwardson and Gilbert, 1976; Pickering and Fink, 1976; 1979); (3) it can regulate LHRH receptor number and; (4) it can induce gonadotrophin synthesis (Fink, 1988). These actions of LHRH are important for maintaining the reproductive cycle in the mammal. In particular, in the female rat *in vivo* LHRH priming acts in co-ordination with the cyclic surge of LHRH in the hypophysial portal blood, producing a massive release of LH, leading to ovulation on a day of the cycle known as pro-oestrus.

1.4.1 Luteinizing hormone-releasing hormone actions on gonadotrophin release

The first evidence for a priming action of LHRH came from *in vivo* studies in pro-oestrous rats in which the LH response to the second of two injections with LHRH was significantly greater than the response to the first LHRH injection (Aiyer *et al,* 1974). In later experiments, LHRH priming was demonstrated in pro-oestrous rat anterior pituitary tissue *in vitro* and a number of major differences between the priming and releasing actions of LHRH were detected (Table 1.1). For example, LHRH-induced LH release, but not LHRH priming, is dependent upon extracellular Ca^{2+} and can be elicited by Ca^{2+} ionophores and depolarising concentrations of K^+ (Pickering and Fink, 1979). Ultrastructural studies have shown that priming is accompanied by a change in orientation and length of the gonadotroph microfilaments and movement of the secretory granules to the plasma membrane. This action may explain why there is an increase in size of the readily releasable pool of LH prior to the LH surge (Fink, 1988). The priming response, but not the releasing action of LHRH, requires synthesis of a protein, other than additional hormone (Edwardson

and Gilbert, 1976; Pickering and Fink, 1976, 1979). This protein may be a 70 kDa, heat-shock protein (Curtis *et al,* 1985), whose exact function in priming has yet to be clarified. Luteinizing hormone-releasing hormone priming is associated with an increased production of IP_3 and facilitated Ca^{2+} mobilisation (Mitchell *et al,* 1988), thus the priming response is associated with an increase in stimulus-secretion coupling. Pre-treatment with PKC activators can augment LHRH-induced hormone release (Turgeon and Waring, 1986), raising the possibility that PKC may modulate LHRH priming. Consistent with this hypothesis, in a preliminary study, staurosporine inhibited the priming actions, but not the releasing effects of LHRH (Fink *et al,* 1990). However, in another study (Johnson *et al,* 1988), H7 was unable to inhibit either the priming or releasing actions of LHRH. Nevertheless, the possibility that PKC activation may be required for LHRH priming requires further investigation.

In vivo, the LH surge is preceded by a surge of oestrogen (E_2) and is accompanied and followed by an increase in plasma progesterone levels. There is good evidence that E_2 is required to maintain the LH surge and that this modulatory action of E_2 is partly due to an indirect effect on the LHRH-releasing neurones, facilitating LHRH release (Fink, 1988). Oestrogen also facilitates LHRH-induced LH release by having direct effects on gonadotroph responsiveness (Drouin *et al,* 1976; Debeljuk *et al,* 1978). This action may be due to an effect of E_2 on the intracellular mechanisms which control gonadotrophin release. For example, long-term E_2 treatment of ovariectomised rats and of cultured anterior pituitary cells can facilitate LH responses to PKC activators measured *in vitro* (Liu and Jackson, 1988; Bourne *et al,* 1989; Fahmy *et al,* 1989), probably by inducing synthesis of additional PKCs (Drouva *et al,* 1990). Progesterone can also enhance LHRH responses, possibly by having effects on gonadotroph stimulus-secretion coupling (Mann and Barraclough, 1973; Turgeon and Waring, 1981, 1990). The ovary releases a number of non-steroidal factors, including inhibin, that have a negative influence on LHRH

responses (de Konig *et al,* 1987; Koppenaal *et al,* 1991). For example, inhibin treatment of rat anterior pituitary cell cultures for 5 days reduced subsequent secretory responses to phorbol esters (Wang *et al,* 1990), suggesting that inhibin may suppress responses to LHRH by having an effect on PKC, or some mechanism subsequent to PKC activation. Thus, both gonadal steroids and non-steroidal factors may modulate gonadotrophin release and LHRH priming by affecting post-receptor signalling events.

1.4.2 The role of calcium in LHRH receptor responses

Preliminary studies suggested that LH release occurred by a Ca^{2+}-dependent mechanism, since Ca^{2+} ionophores could induce LH release from pituitary cultures (Conn *et al,* 1979; Naor and Eli, 1985; Chang *et al,* 1986b), LHRH-induced LH release coincided with an increase in $^{45}Ca^{2+}$ flux from pre-loaded anterior pituitary pieces (Williams, 1976) and secretory responses to LHRH were, in part, dependent upon the presence of extracellular Ca^{2+} (Marian and Conn, 1979). More recent studies have shown that LHRH induced inositol phospholipid breakdown both in dispersed anterior pituitary cells (Snyder and Bleasdale, 1982) and in cell populations enriched in gonadotrophs (Raymond *et al,* 1984; Andrews and Conn, 1986) and that this was followed by an increase in $Ins(1,4,5)P_3$ and DAG accumulation (Schrey, 1985; Andrews and Conn, 1986). Inositol (1,4,5) trisphosphate production and Ca^{2+} mobilisation may, therefore, be a major pathway for LHRH-induced LH release. Since G-protein analogues induced an increase in inositol phosphate production and LH release from ATP-permeabilized dispersed rat anterior pituitary cells (Andrews *et al,* 1986), the LHRH receptor is probably coupled to PLC via a G-protein.

In cultured anterior pituitary cells, the pattern of LHRH-induced gonadotrophin release is biphasic, consisting of an initial rapid peak of hormone release within the first 10 minutes of stimulation, followed by a more sustained

release (Borges *et al,* 1983; Tasaka *et al,* 1988). This temporal pattern of hormone release correlated with changes in the requirement of the response for Ca^{2+} from intracellular and extracellular sources. Using fluorescent indicators to measure cytosolic free Ca^{2+} levels in single cell populations of enriched gonadotrophs, LHRH stimulation was shown to induce an initial spike of Ca^{2+} which is followed by a more sustained increase in Ca^{2+} (Clapper and Conn, 1985). Removal of the extracellular Ca^{2+} reduced the initial Ca^{2+} spike by 35 - 60% and prevented the prolonged increase in intracellular Ca^{2+} (Chang *et al,* 1986b; Limor *et al,* 1987; Tasaka *et al,* 1988). Thus, approximately half of the spike phase of Ca^{2+} release comes from intracellular stores, the remaining Ca^{2+} apparently entering from extracellular sources through dihydropyridine (DHP)-insensitive Ca^{2+} channels (Smith *et al,* 1987; Tasaka *et al,* 1988). Calcium entry through the DHP-insensitive channels continues into the later phase of LH release, during which, Ca^{2+} entry (approximately 30% of the later phase) also occurs through DHP-sensitive Ca^{2+} channels (Chang *et al,* 1986b; Shangold *et al,* 1988).

The LHRH-mediated increase in intracellular Ca^{2+} levels may induce gonadotrophin release by modulating the activity of a number of different Ca^{2+}-dependent processes. In particular, LHRH treatment of ovariectomized rats causes a redistribution of calmodulin from the cytosol to the plasma membrane (Conn *et al,* 1981a), a process which may have functional significance since the activities of calmodulin-dependent enzymes may be altered. Indeed, a component of LHRH-induced LH release can be inhibited by calmodulin antagonists (Conn *et al,* 1981b), suggesting a role for Ca^{2+}/calmodulin-dependent enzymes in this process.

1.4.3 The role of protein kinase C in LHRH receptor responses

Although LHRH receptor activation also liberates DAG (Andrews and Conn, 1986) which can activate PKC (section 1.2), the involvement of PKC in LHRH responses is controversial (see Conn, 1989 for review). Luteinizing hormone-

releasing hormone can induce PKC translocation to the cell membrane (Hirota *et al,* 1985; Naor *et al,* 1985; McArdle and Conn, 1986), with a time-course and dose-dependency similar to that of LHRH-induced LH release. Furthermore, DAGs and phorbol esters induced gonadotrophin release from anterior pituitary cells in culture (Smith and Vale, 1980; Conn *et al,* 1985; Nigro-Villar and Lapetina, 1985; Chang *et al,* 1987b) and anterior pituitary pieces *in vitro* (Turgeon and Waring, 1986; Johnson and Mitchell, 1989). Introduction of purified rat brain PKC into permeabilized anterior pituitary cells also induced LH release (Naor *et al,* 1989), consistent with a role for PKC in LH release responses. Notably, Ca^{2+} ionophores and PKC activators mimicked the early and late secretory responses to LHRH respectively (Harris *et al,* 1985; Naor and Eli, 1985; Turgeon and Waring, 1986; Chang *et al,* 1987b). Although some studies show that LHRH-induced LH release, and the later secretory response in particular, are prevented by PKC inhibitors (Hirota *et al,* 1985; Chang *et al,* 1987b; Stojilkovic *et al,* 1991), others have reported that LHRH responses are not prevented by these drugs (van der Merwe *et al,* 1990b). Thus, further studies are required to clarify the role of PKC in LHRH response.

Attempts to elucidate the involvement of PKC in LHRH responses by phorbol ester-induced down regulation of pituitary PKC levels have also produced conflicting results. For example, after down regulation of PKC in dispersed anterior pituitary cells, some have reported that both LHRH and phorbol ester secretory responses are attenuated (Stojilkivic *et al,* 1988b, 1991). In contrast, similar studies show that, although phorbol ester-induced hormone release is reduced, the response to LHRH is unaffected (McArdle and Conn, 1986; McArdle *et al,* 1987). The discrepancies between these down-regulation studies may be accounted for by differences in the down-regulation protocols. For example, in a study by McArdle *et al* (1987), anterior pituitary PKC levels were down regulated by PMA treatment for 6 hours, a protocol which can down-regulate PKC levels by only approximately 50% (Stojilkovic *et al,* 1991). It is likely that the PKC(s) involved in LHRH responses

may not be down-regulated completely by this method. In another study (Stojilkovic *et al,* 1991), anterior pituitary cells were treated with PMA over a longer time-scale (24 hours), during which, PKC was down regulated by 90% and LHRH secretory responses were attenuated. Several problems can arise from using chronic phorbol ester treatment as a method of down-regulating PKC. Firstly, since PKC can phosphorylate a number of different cellular targets, prolonged phorbol ester treatment may causes long-term changes in cell function, which can alter cell responsiveness. In addition, phorbol esters can release LH and, on sustained application, may reduce gonadotrophin levels by 70 - 80% (Stojilkovic *et al,* 1988a, 1988b). Ideally, gonadotroph PKC levels should be down-regulated under conditions that will not affect gonadotrophin secretory capacity. As a consequence, PKC down-regulation by prolonged phorbol ester treatment is not an ideal system in which to study PKC involvement in LHRH responses.

In a large number of studies, dispersed anterior pituitary cells, maintained in culture, have been used to examine LHRH action. Although cultured cells respond to LHRH, it is clear that they function differently than tissue *in vivo* and to anterior pituitary pieces incubated *in vitro.* For example, LHRH can induce LH biosynthesis in gonadotrophs *in vivo* and in tissue pieces incubated *in vitro,* but is unable to do so in cultured cells. In addition, LHRH is unable to induce a priming response in dispersed cells (Speight and Fink, 1981; Chang *et al,* 1987b) whilst priming does occur in tissue pieces (Edwardson and Gilbert, 1976; Pickering and Fink, 1976, 1979; Turgeon and Waring, 1981). There are several reasons why cultured cells may not function in an entirely physiological way. First of all, it is clear that steroids can have modulatory effects on a number of factors which may contribute to LHRH responses and LHRH priming in particular. Oestrogen, for example, can influence PKC expression in cultured anterior pituitary cells, enhancing LH secretory responses to phorbol esters (Drouva *et al,* 1990) and LHRH (Audy *et al,* 1990). Therefore, it is possible that any PKCs which function in the LHRH signal may be down-regulated in

cells which have been cultured in steroid-free conditions, explaining why a number of workers have been unable to find any PKC involvement in LHRH receptor signalling using this system. Secondly, enzymatic dispersion may cause changes in LHRH receptor properties (Naor *et al,* 1980). Thirdly, dispersed pituitary cells are incubated in the absence of LHRH, whereas *in vivo* the pituitary is under the continual influence of LHRH pulses. Finally, gonadotrophs are suggested to specifically associate with lactotrophs via gap junctions (Nakane *et al,* 1970) and physiological interactions, such as the exchange of inorganic and organic molecules, may take place between these cell types (Denef and Andres, 1983). Dispersed cells in culture, therefore, are not an ideal system for studying the involvement of PKC (or, indeed, other cellular signals) in LHRH responses.

Anterior pituitary tissue pieces and cells in culture consist of populations of different cell types, only approximately 10% of which are gonadotrophs and unless a cell specific response, such as LH/FSH release, is measured, problems may arise with regards to interpreting certain studies. Some groups have carried out studies using gonadotrophs from dispersed anterior pituitaries which have been purified by elutriation (Raymond *et al,* 1984; Hirota *et al,* 1985; Andrews and Conn, 1986). An alternative approach has been to use clonal cell lines but, until very recently, no gonadotroph cell line existed. However, recently, Mellon and her colleagues have produced a clonal cell line from transgenic mice, the α T3-1 cell line, which expresses high affinity LHRH receptors (Windle *et al,* 1990; Horn *et al,* 1991). Like dispersed anterior pituitary cells, α T3-1 cells mobilise intracellular and extracellular Ca^{2+} in response to LHRH (McArdle *et al,* 1992). However, these cells do not synthesis or secrete the β LH or FSH subunits (Windle *et al,* 1990), limiting their usefulness as a model of LHRH action in gonadotrophs. Although future development of similar cell lines, which function more physiologically, may help to characterise the molecular mechanism of LHRH action, these cell lines, like dispersed cells, will probably suffer from the drawbacks described above. Of all of the

described systems, anterior pituitary tissue pieces incubated *in vitro* seem to function most similarly to anterior pituitaries *in vivo* and are therefore, a more physiological system in which to characterise the role of effectors, such as PKC, in LHRH responses.

1.4.4 The role of arachidonic acid and it metabolites in LHRH responses

Arachidonic acid and its lipoxygenase and epoxygenase metabolites may have a second messenger function in LHRH receptor signalling. Using cultured anterior pituitary cells, AA can induce LH release and LHRH can induce an increase in [^{3}H]-AA release from pre-labelled cells (Naor and Catt, 1981; Chang *et al,* 1986a, 1987b). Addition of melittin or exogenous PLA_2 can induce LH release from cultured anterior pituitary cells (Kiesel *et al,* 1985). The PLA_2 inhibitor, quinacrine, can inhibit 40% of the LH/FSH secretory response to LHRH; the remaining release of hormone being blocked by the DAG lipase inhibitor, RHC 80267 (Chang *et al,* 1988). These results suggest that LHRH may induce AA release by a route involving both DAG lipase and PLA_2 action. The mechanism by which LHRH receptors couple to PLA_2 and DAG lipase is poorly understood.

Luteinizing hormone-releasing hormone-induced LH release from dispersed anterior cells is prevented by lipoxygenase inhibitors, including NDGA and BW 755C and by the more selective lipoxygenase inhibitors, L-656,224 and MK 886, but not cycloxygenase inhibitors (Naor and Catt, 1981; Naor *et al,* 1983; Dan-Cohen *et al,* 1992) suggesting a role for lipoxygenase metabolites as mediators of LHRH responses. Consistent with this hypothesis, LHRH induces an increase in leukotriene production (Vanderhoek *et al,* 1984; Kiesel *et al,* 1991). Different lipoxygenase metabolites have been shown to induce LH release from dispersed anterior pituitary cells, but with conflicting results. 5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) can induce LH release from cultured cells, but at concentrations which are unlikely to be physiological (> 1 μM) (Naor *et al,* 1983; Kiesel *et al,* 1987). In some

investigations, LTC_4 and LTB_4 have been shown to induce LH release at concentrations which may be physiologically relevant (Catt *et al,* 1985; Naor *et al,* 1985; Dan-Cohen *et al,* 1992). However, a second report suggests that LTC_4 only is effective (Hulting *et al,* 1985) and yet a third report indicates that neither LTC_4 nor LTB_4 can induce LH release (Naor *et al,* 1983). Clearly, these results do not provide a definitive conclusion as to the role of specific lipoxygenase metabolites in LHRH responses. Nevertheless, leukotriene receptor antagonists, compounds like FPL 55,712, ICI 198 615 and LY 171 883, can partially inhibit LHRH-induced LH release from dispersed rat anterior pituitary cells at concentrations where they are known to selectively antagonise LTD_4, LTC_4 and LTE_4 receptor actions (Kiesel *et al,* 1991; Dan-Cohen *et al,* 1992). It would seem, therefore, that lipoxygenase metabolites, and certain leukotrienes in particular, may play a role, at least in part, in the mechanism of LHRH-induced LH release from dispersed rat anterior pituitary cells.

The epoxygenase metabolite 5,6-epoxyeicosatrienoic acid can also induce LH release from dispersed anterior pituitary cells but at a concentration (100 nM) that may not be physiological (Snyder *et al,* 1983). However, these experiments do not discount the possibility that certain epoxygenase metabolites may function in the process of LHRH-induced LH release. Additional experiments examining the effect of epoxygenase inhibitors on secretory responses to LHRH will be of interest since they may clarify the role of these AA metabolites in the LHRH receptor signal.

1.5 THE AIM OF THIS STUDY

The aim of this research project has been to elucidate the pharmacology, cellular targets and physiological actions of PKC forms in rat anterior pituitary tissue, particularly in the context of the priming effect of LHRH. Thus, a number of questions were addressed: (1) is PKC activation required for gonadotrophin release in response to LHRH and/or the LHRH priming effect? (2) if so, what are the pharmacological profiles of the PKC forms that control these responses? (3) is PLA_2

a cellular target of these PKC forms? (4) do gonadal steroids, such as oestrogen, modulate responsiveness to LHRH by having an effect on these PKC forms and/or their targets?

FIGURE 1.1

Phospholipid breakdown by different forms of phospholipase

Agonist-stimulated phospholipid breakdown can occur by the actions of one or more type of phospholipase. Both phospholipase C (PLC) and phospholipase D (PLD) catalyse the hydrolysis of phosphodiester linkages. The catalytic actions of PLC would release 1,2-diacylglycerol and the polar head group of the phospholipid, whereas PLD-induced phospholipid hydrolysis would liberate phosphatidic acid and a free base (depicted here as R_2). The R_2 position base may be either inositol or choline. Phospholipase A_2 (PLA_2) catalyses the hydrolysis of the *sn*-2 bond of the phospholipid backbone, releasing free fatty acid, such as arachidonic acid (as shown here), and lysophospholipid, containing a fatty acid at the *sn*-1 position (R_1).

Figure 1.1

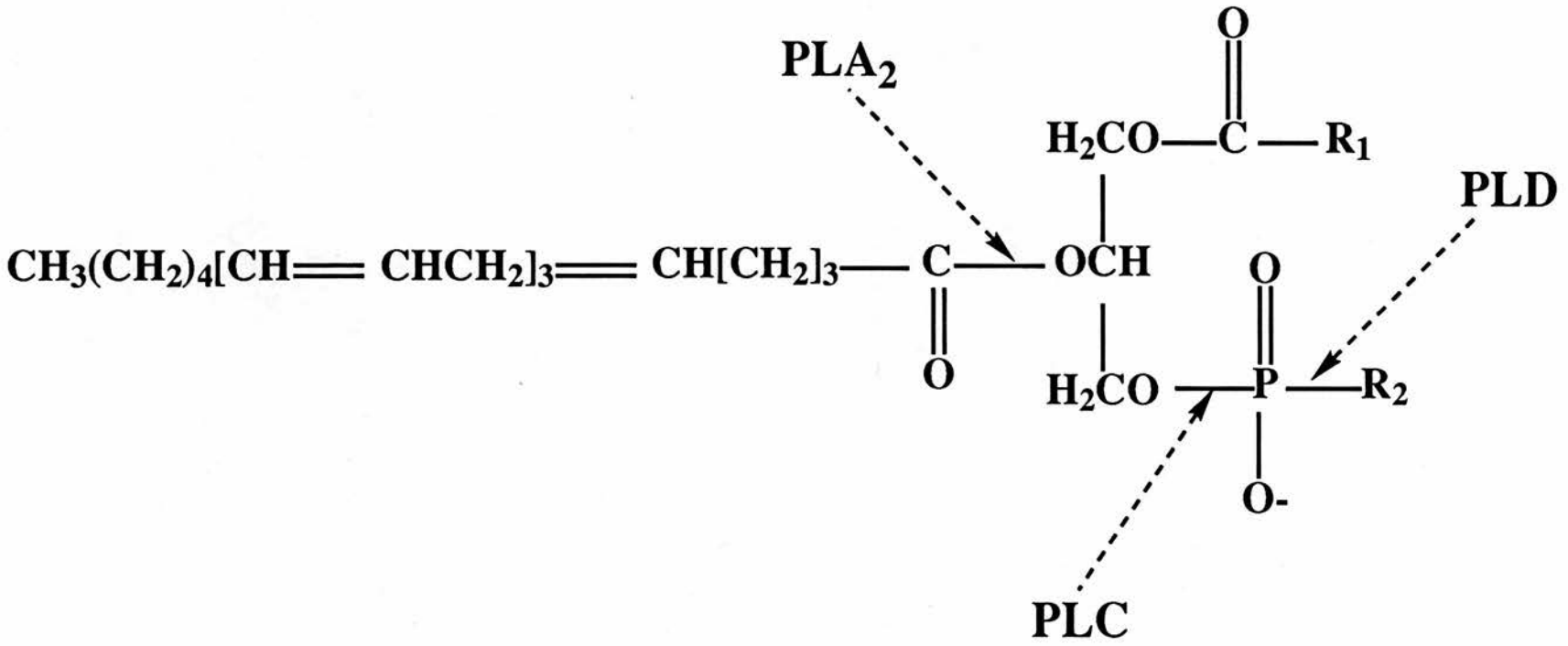

PLA2
O
H2CO—C—R1
PLD
CH3(CH2)4[CH═ CHCH2]3═ CH[CH2]3—C—OCH
O
O
H2CO—P—R2
O-
PLC

Figure 1.2

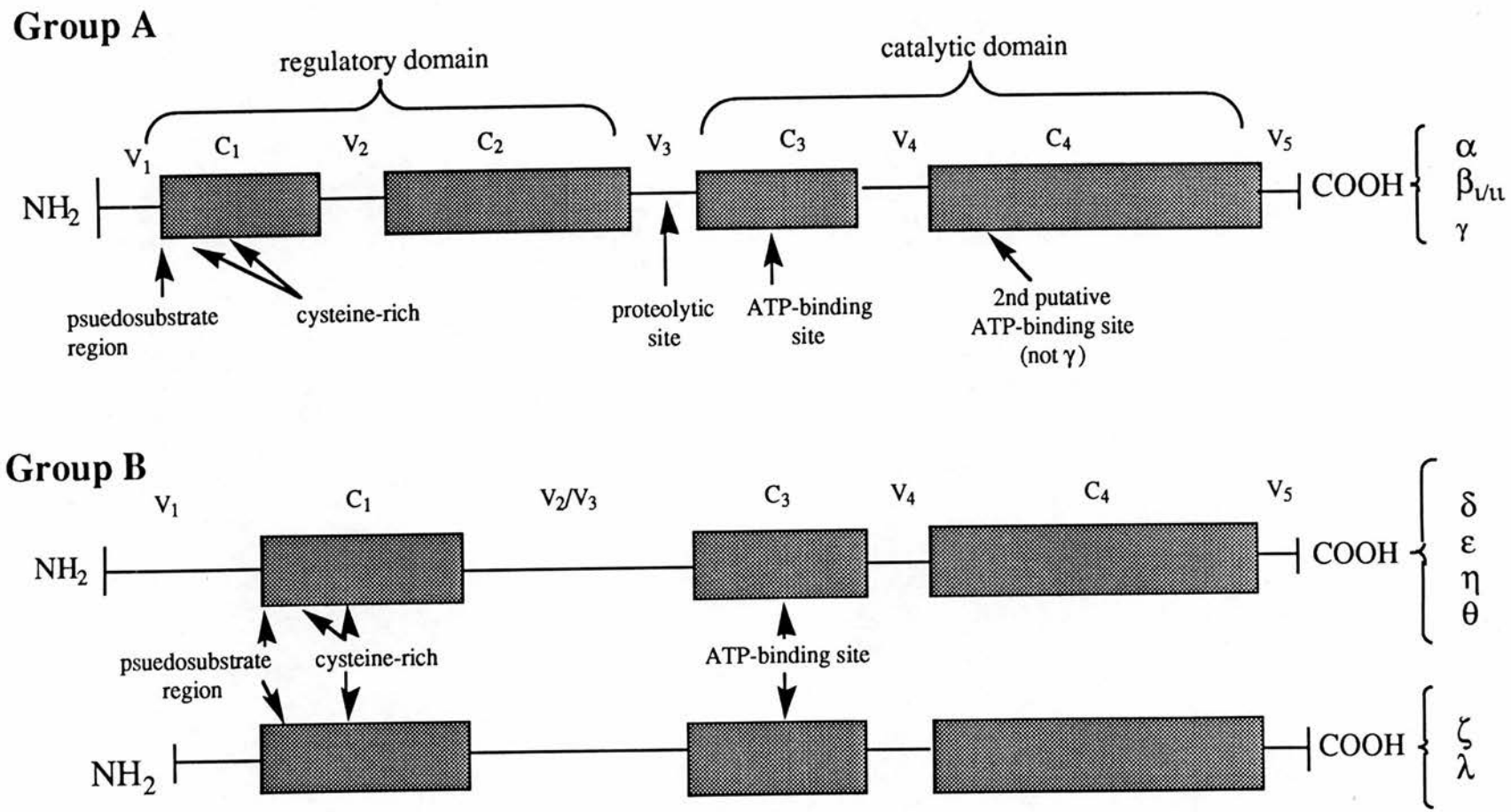

Group A
regulatory domain
catalytic domain
V1
C1
V2
C2
V3
C3
V4
C4
V5
NH2
COOH
α
βI/II
γ
psuedosubstrate region
cysteine-rich
proteolytic site
ATP-binding site
2nd putative ATP-binding site (not γ)
Group B
V1
C1
V2/V3
C3
V4
C4
V5
NH2
COOH
δ
ε
η
θ
psuedosubstrate region
cysteine-rich
ATP-binding site
NH2
COOH
ζ
λ

FIGURE 1.2

Protein kinase C enzyme family

This figure is a schematic representation of the cDNA sequences of the known PKC isoforms. The A series (α, β and γ) are the Ca^{2+}-dependent forms whereas the B series (γ, ε, ζ, η, θ and λ) are the Ca^{2+}-independent forms. The boxed regions represent the conserved regions of PKC (C_1 - C_4) whilst the variable regions are shown by V_1 - V_5. Analysis of the functions of these domains has been based upon deletion/mutagenesis studies, construction of chimeric PKCs, sequence analysis and by making biochemical comparisons with other enzymes. This figure has been adapted and updated from one presented by Huang (1989).

Figure 1.3

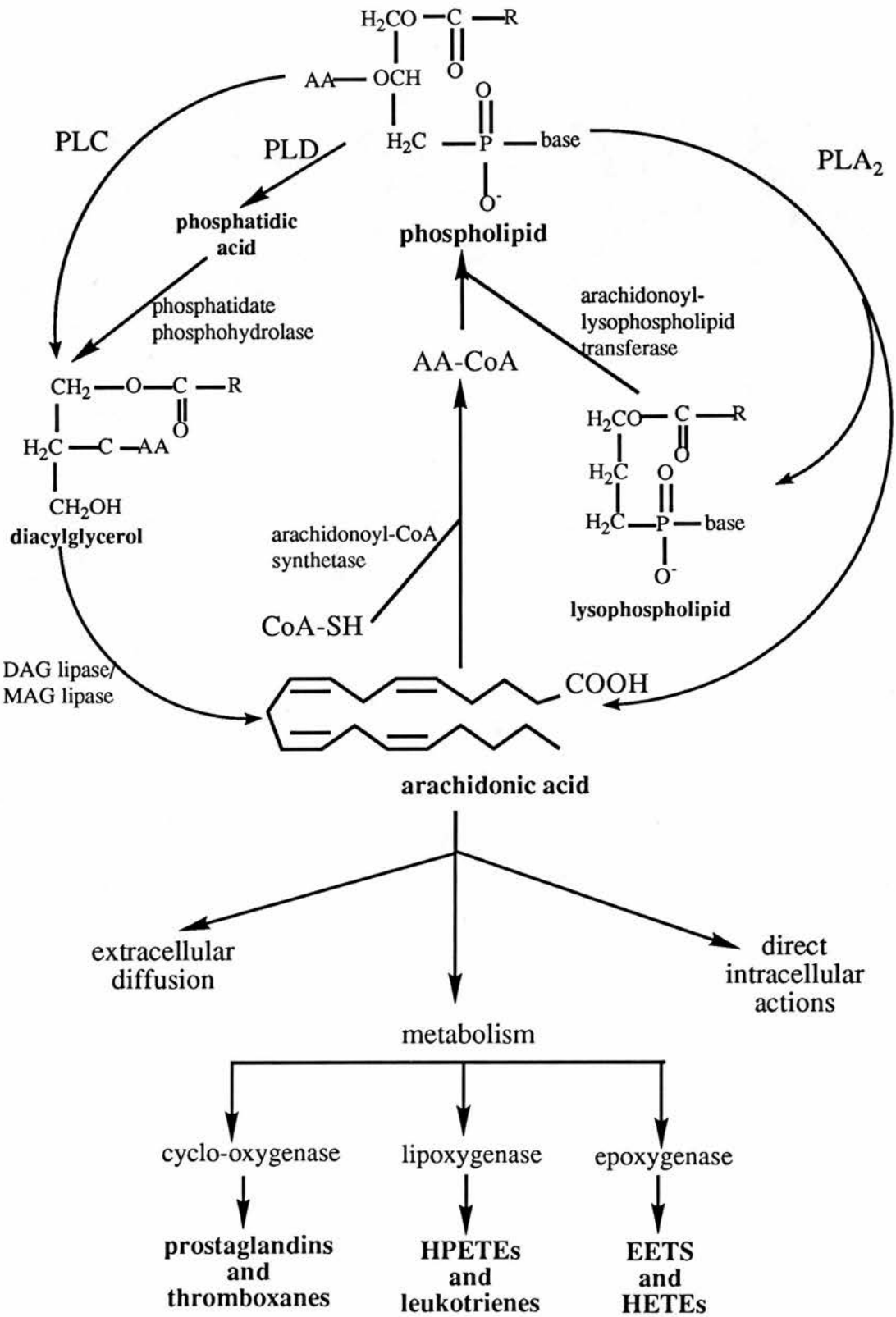

PLC
PLD
PLA2
phosphatidic acid
phospholipid
phosphatidate phosphohydrolase
arachidonoyl-lysophospholipid transferase
AA-CoA
diacylglycerol
arachidonoyl-CoA synthetase
CoA-SH
lysophospholipid
DAG lipase/ MAG lipase
COOH
arachidonic acid
extracellular diffusion
direct intracellular actions
metabolism
cyclo-oxygenase
lipoxygenase
epoxygenase
prostaglandins and thromboxanes
HPETEs and leukotrienes
EETS and HETEs

TABLE 1.1

Comparison between the releasing and priming actions of LHRH in the pro-oestrous rat

(Based on Fink (1986)).

	Releasing	Priming
Can be repeated frequently	Yes	No (only once in 3 h period)
Enhanced by oestrogen	Yes	Yes
Dependent on extracellular Ca^{2+}	Yes	No
Mimicked by high K^{+}	Yes	No
Mimicked by Ca^{2+} ionophores	Yes	No
Dependent on protein synthesis	No	Yes
Dependent on integrity of microfilaments	No	Yes
Elicited in dispersed cells	Yes	Not for rat pituitary glands
Cyclic AMP acts as a mediator	No	No
Ins (1,4,5)P_3 acts as a mediator	Yes	?
PKC acts as a mediator	?	?
AA and/or its metabolites act as mediators	?	?

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

[5,6,8,9,11,12,14,15-^{3}H(N)]-Arachidonic acid (specific activity approximately 240 Ci/mmol), adenosine 5'-[γ-^{35}S-thio]trisphosphate (ATP-γ-^{35}S) (specific activity approximately 240 Ci/mmol) and [20-^{3}H(N)]-phorbol 12,13-dibutyrate (specific activity = 19 Ci/mmol) [^{3}H]-N,N-dimethylstaurosporine (specific activity = 160 Ci/mmol) were purchased from Du Pont, Dreieich, Germany. Luteinizing hormone-releasing hormone (LHRH), phorbol 12,13-dibutyrate (PDBu), 1,2-dioctanoyl-*sn*-glycerol (DOG), mezerein, quinacrine dihydrochloride, *p*-bromophenacyl bromide (BrPheBr), 4, 8, 11, 14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), indomethacin, arachidonic acid sodium salt (AA), arachidic acid, oestradiol 17β (E_2), bovine serum albumin (BSA; essential fatty acid free), deoxyribonuclease, hyaluronidase, docosahexaenoic acid, penicillin, streptomycin, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64), 2-mercaptoethanol (EtSH), phenylmethylsulphonyl fluoride (PMSF), lysine-rich histone IIIS, Dulbecco's modification of Minimal Essential Medium powder (without phenol red, but with 1000 mg/ml glucose and l-glutamine) were all purchased from Sigma Chemical Company Ltd, Poole, Dorset, UK. K252a was purchased from Kyowa Medex Co, Tokyo, Japan, ionomycin, staurosporine and Nonidet P-40 were purchased from Novabiochem, Nottingham, UK and aristolochic acid sodium salt was purchased from Biomol Research Laboratories, c/o Semat, St Albans, Herts, UK. 4-Chloro-*N*-(*p*-pentylcinnamoyl) anthraniric acid (ONO-RS-082) was a gift from ONO Pharmaceuticals, Osaka, Japan. Ro 31-8220, Ro 31-4639 and Ro 31-4493 were gifts from Roche Products Ltd, Welwyn Garden City, Herts, UK. 1,6-Di(*O*-(carbamoyl) cyclohexaneoxime) hexane (RHC 80267) was a gift from Wellcome Research Laboratories, Beckenham, Kent, UK. Foetal calf serum was purchased from Sera Lab, Crawley Down, Sussex, UK. Leupeptin and collagenase were obtained from Boehringer Mannheim, Lewes, UK and phosphatidylserine (sodium salt) from Lipid

Products, Nutfield, Surrey. Hepes-buffered minimal essential medium containing Earle's salts (MEM), Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate and glutamine, Earle's balanced salt solution (EBSS) with magnesium but without phenol red and 1-(5-isoquinolinesulphonyl-2 methylpiperazine) dihydrochloride (H7) were obtained from Gibco Brl, Paisley, Scotland, UK.

Analar grade laboratory chemicals were obtained from BDH, Dagenham, Essex, UK. COB-Wistar rats were purchased from Charles River UK Ltd, Margate, Kent, UK or were obtained from a colony bred in this department which were derived from Charles River Wistar rats. The α T3-1 cell line was a gift from Dr Pamela Mellon, the Salk Institute, USA. Radioimmunoassay materials were gifts from Dr S Raiti of the NHPP, University of Maryland School of Medicine, Baltimore, MD, USA, Drs GD Niswender, LE Reichert Jr and the Pituitary Hormone Distribution Agency of the NIADKK, Baltimore, MD, USA and the Scottish Antibody Production Unit, Carluke, Lanarkshire, UK.

2.2 METHODS

2.2.1 Animals

Adult female (~ 200 g) or male (~ 250 g) COB Wistar rats were maintained under controlled lighting (lights on from 05.00 to 19.00 h) and temperature (22°C) with free access to food pellets (CRM, Labsure, Manea, Cambs, UK) and tap water.

Female rats were assessed for a minimum of two 4 day oestrous cycles (as determined by vaginal lavage) before being anaesthetised with Sagatal (30 mg ml/kg body weight; sodium pentabarbitone; May and Baker, Dagenham, Essex) by 11.00 - 11.30 am on the appropriate day of the cycle. The cytological characteristics of the vaginal smears in 4-day cyclic rats were as follows;

Metoestrus: large numbers of leucocytes, epithelial cells and cornified epithelial cells.

Dioestrus: leucocytes, epithelial cells and cornified epithelial cells, but in smaller amounts than metoestrous animals.

Pro-oestrus: mainly nucleated epithelial cells.

Oestrus: predominantly cornified epithelial cells.

To study the effects of oestrogen (E_2) treatment on anterior pituitary responsiveness, female rats, at random stages of the oestrous cycle, were bilaterally ovariectomised under halothane anaesthesia and left over a period of 4 weeks before being randomly divided into two experimental groups. The E_2-treated group received subcutaneous implants in the dorsal neck with 1 cm long silicone elastomer capsules (Dow-Corning Corporation, Michigan, USA), with an internal diameter of 0.078 inches and an external diameter of 0.125 inches, containing 0.5 cm of crystallised oestradiol 17β and the control (i.e. E_2 untreated) group received equivalent implants but with empty capsules (Dzuik & Cook, 1966; Henderson *et al,* 1977). On the morning of the 5th day after implantation, vaginal smears were examined. Ovariectomised animals which had been implanted with empty capsules had smears similar in characteristic to those seen dioestrus whereas those animals which had been implanted with E_2-containing capsules had smears similar to those seen on oestrus. Animals were anaesthetised and decapitated by 11.30 am on the 5th day after implantation.

2.2.2 Anterior pituitary cell dispersion and cell culture

Anterior pituitary pieces were removed from 5 - 6 female rats using, as far as possible, sterile technique and placed into a sterile petri dish containing approximately 5 ml of sterile Earle's balanced salts solution (EBSS) and cut into 8 pieces of approximately equal size. The tissue pieces were then transferred to a second sterile petri dish containing 5 ml of sterile EBSS containing 0.2% collagenase, 0.2% hyaluronidase and 2 μg/ml deoxyribonuclease. The tissue was then left to incubate with the enzymes for 1 hour at 37°C under an atmosphere of 95% air/5%

CO_2. The cells were dispersed by tritutating the enzyme-treated tissue with a sterile glass pasteur pipette. The cells were separated from the enzyme solution and cell debris by centrifugation (room temperature, 750 g, 10 min). The supernatant was discarded and the pellet was resuspended in 5 ml of EBSS containing 2 μg/ml deoxyribonuclease. The cell suspension was pipetted onto fibronectin-treated 12 well plates (0.2 ml/well) along with 1 ml of pre-warmed DMEM (made from DMEM powder as described in Appendix I.2) containing 10% steroid-stripped foetal calf serum (see Appendix I.1), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated for 4 days in a humified atmosphere of 95% O_2/5% CO_2 at a constant temperature of 37°C, after which, the medium was replaced.

α T3-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate (110 mg/l) containing 10% foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, under a humidified atmosphere of 95% air/5% CO_2 at 37°C and received fresh medium every 3 - 4 days. Cells were allowed to grow to 100% confluency and were split and either seeded, at low density, in fresh flasks or in 12 well plates for assay purposes. Cells were harvested by trypsin digestion; the medium was removed and replaced with 5 ml of 0.25% (v/v) trypsin solution (37°C). After 15 - 30 seconds, the trypsin solution was removed and the flasks were incubated for a further 5 minute period (37°C). Cells were detached from the culture flask by agitation in fresh DMEM. The cell suspension was split into new flasks.

For storage, harvested cells were resuspended in 95% foetal calf serum + 5% dimethylsulphoxide (~ 10^7 cells/ml) and aliquoted into cryostat tubes, which were then sealed. The cells were frozen at -70°C for 3 - 5 h before being placed in liquid N_2. Cells were recovered from storage by placing the frozen cryostat tube into a beaker of water (37°C) for 2 minutes. The defrosted cell suspension was transferred into a flask containing pre-warmed (37°C) DMEM using a sterile syringe and wide gauge needle.

All tissue culture was carried out using sterile apparatus and in sterile flow hoods.

2.2.3 Hormone secretion experiments

The methods were based upon those used by Pickering and Fink (1976) (Figure 2.1). Anterior pituitary glands were hemisected and randomly distributed into 25 ml conical flasks containing 2 ml of pre-gassed and pre-warmed (37°C) Hepes-buffered minimal essential medium with Earle's salts (MEM). The hemipituitary pieces were pre-incubated for 30 min in a shaking water bath at 37°C under an atmosphere of 95% O_2/5% CO_2, after which, the medium was discarded and replaced hourly with fresh pre-warmed and pre-gassed MEM. To examine the actions of various secretagogues on hormone release, the tissue was incubated for an initial, basal hour in the presence of MEM only, followed by consecutive hours in the presence of either LHRH, a PKC activator or ionomycin as appropriate. In the experiments where the effects of PKC/PLA_2 inhibitors were examined, the inhibitor was present in the basal hour as well as the 1st, 2nd and 3rd hours, together with the appropriate secretagogue. The medium was collected at the end of each hourly incubation and was stored at -20°C until it was radioimmunoassayed for LH, FSH or GH (Niswender *et al,* 1968; Daane and Parlow, 1971, see Appendix II). Where drugs were made up as stock solutions in dimethylformamide (DMF) or ethanol, the maximum concentration of each solvent was used in control experiments.

The mean intra- and inter-assay variables in the LH and GH radioimmunoassays were less than 5% and 7% respectively. Luteinizing hormone was assayed using either NIH-LH-S18 or NIADDK-rat LH-RP2 and data were normalised for comparison. Other minor factors which may affect the reproducibility of the experimental data include small differences between pituitaries, such as minimal variations in size.

2.2.4. Cytosolic protein kinase C activity assay

Anterior pituitary PKC activity was determined as the phosphatidylserine-dependent, histone IIIS kinase activity induced by PKC activators using methods modified from those described by Wise *et al* (1982) and Huang *et al* (1988) (Figure 2.2). Anterior pituitary tissue was homogenised in 2 volumes (volume = 1ml/g of tissue, wet weight) of 20 mM Tris HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.01% (w/v) leupeptin and 20 μM E64. The homogenate was centrifuged (16,000 g, 20 min, 4°C) and the supernatant was collected and recentrifuged (16,000 g, 5 min, 4°C). The supernatant from the second spin was taken to represent cytosol and was partially purified by loading onto 1.5 ml DE52 diethylaminoethyl (DEAE) cellulose (Whatman International Ltd, Maidstone, Kent, UK) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, CA, USA) at 4°C. The cytosol was then washed with 6 column volumes of homogenisation buffer before eluting the partially purified PKC with 3 column volumes of buffer containing 150 mM NaCl. Partially-purified cytosolic PKC activity was measured in an assay mixture containing (final concentrations): 12.5 mM $MgCl_2$, 100 μg/ml phosphatidylserine (sodium salt) + 0.04% Nonidet P-40, 1.25 mg/ml histone IIIS, 100 μM ATP-γ-^{35}S (0.595 μCi/tube) and cytosol (final volume = 100 μl). All assay components and drugs were dissolved in 20 mM Tris HCl (pH 7.5) + 0.5 mM EGTA. Assay tubes additionally contained either 600 μM $CaCl_2$ (100 μM free Ca^{2+}) or 5 mM ethyleneglycol-bis-(β-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA) to give Ca^{2+}-dependent and Ca^{2+}-independent conditions respectively . Phosphatidylserine vesicles were prepared by drying the lipid from chloroform/ methanol under a stream of N_2. The subsequent film of phosphatidylserine was scraped into 20 mM Tris HCl (pH 7.5) + 0.5 mM EGTA at a stock concentration of 400 μg/ml, sonicated, then 0.16% Nonidet P-40 was added. The mixture was vortexed before use. Protein kinase C activity was measured either in the absence of

activator (i.e. basal activity) or in the presence of 1 μM PDBu or 100 μM DOG or in the presence of activator and an appropriate PKC inhibitor. DOG which was added to each assay tube in 1 μl DMF using a Hamilton microsyringe. Reactions were carried out at 30°C for 15 min and were stopped by quenching with 20 μl of 0.1 M ATP in 0.1 M EDTA (pH 7.0). The quenched reaction mixture (50 μl) was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange paper (Whatman International Ltd) and washed (3 x 10 ml, 2 min, room temperature) in 75 mM H_3PO_4.

Kinase activity measured in the presence of phosphatidylserine, 5 mM EGTA (i.e. low free Ca^{2+}) and in the absence of PKC activator was taken to represent basal activity and was subtracted from all activator-stimulated levels. Calcium-independent kinase activity was taken to be the activity measured in the presence of 5 mM EGTA and PKC activator. The additional kinase activity measured with samples containing $CaCl_2$ + PKC activator was taken to represent calcium-dependent kinase activity. ATP-γ-^{35}S (rather than ATP-γ-^{32}P) was used because, whilst it is still a good substrate for many types of protein kinase (Eckstein, 1985), the thiophosphorylated products are more resistant to phosphatase action (Coyne *et al,* 1987).

2.2.5 [^3H]-Arachidonic acid release measurements

The release of [^3H]-arachidonic acid ([^3H]-AA) from pre-labelled anterior pituitary tissue or dispersed cells in culture was measured by reverse-phase liquid chromatography on octadecyl silica cartridges (ODS) (sep-pak C_{18} cartridge, Waters Chromatography, Watford, Hertfordshire, UK) using the solvent system described by Powell (1982) (Figure 2.3). Anterior pituitary glands were removed, hemisected and each hemipituitary cut into two equal quarters. Pairs of pituitary quarters were then placed into a silanised flask containing 1 ml of pre-warmed (37°C) and pre-gassed (95% O_2/5% CO_2) MEM and pre-incubated in a shaking water bath (37°C, 95% O_2/5% CO_2) for 30 minutes. The medium was then replaced with fresh MEM

containing 0.5 μCi of [^{3}H]-AA and the tissue incubated with the label for 2 hours. The tissue was then washed three times in MEM containing 1% fatty acid-free bovine serum albumin (BSA) to remove unesterified [^{3}H]-AA.

Dispersed anterior pituitary cells or α T3-1 cells were cultured in 12 well plates for 4 days as described in section 2.2.2. The culture medium was removed and replaced with fresh pre-warmed MEM containing 0.5 μCi of [^{3}H]-AA. The cells were incubated for 18 hours in an incubator at 37°C under a humidified atmosphere of 95% air/5% CO_2. The cells were then washed three times in MEM containing 1% BSA.

For studies of [^{3}H]-AA release, after washing, the tissue or cells were incubated in MEM + 0.5% BSA (as a trap for released [^{3}H]-AA) containing either no drug (basal) or an appropriate stimulus. After 15 min, the incubation medium was removed and placed in 2 ml ice-cold ethanol, and 3.7 ml of distilled H_2O was added (to give a final ethanol concentration of 30%) and this was retained for [^{3}H]-AA determination. The cells/tissue were washed twice in pre-warmed and pre-gassed MEM + 1% BSA then a final 1 ml aliquot of pre-warmed and pre-gassed MEM + 0.05% BSA was added to each. The cells were scraped into the medium. The tissue pieces/cells suspension were transferred to a silanised hand held tissue grinder along with the medium and homogenised. The homogenate was added to 2 ml of ice-cold ethanol and was then diluted with distilled water (3.7 ml) to give a final ethanol concentration of 30%.

Both tissue/cell homogenates and medium were centrifuged (10', 5°C, 3000 g) and the supernatant from each was retained and acidified to pH 3 with 1M HCl. To measure medium and tissue [^{3}H]-AA levels, a 4 ml aliquot of the acidified supernatant from each was loaded onto an ODS column (pre-washed with 5 ml ethanol followed by 5 ml of dH_2O). Solvents were then passed through the columns in the following order: (a) 30% ethanol (20 ml) to elute polar substances such as polar lipids; (b) distilled H_2O (20 ml) to remove the ethanol; (c) petroleum ether (10

ml) to remove the water; (d) petroleum ether: $CHCl_3$ (1:1, 20 ml) to elute fatty acids; (e) methyl formate (10 ml) to elute prostaglandins and leukotrienes; (f) 80% ethanol (20 ml); (g) 100% ethanol (20 ml); and (h) distilled H_2O (20 ml) to regenerate the matrix. A 4 ml aliquot of the petroleum ether: $CHCl_3$ fraction was allowed to evaporate off overnight, then the radioactivity in 4 ml aliquots of each solvent fraction was counted by liquid scintillation counting. The radioactivity in 200 µl samples of the acidified tissue homogenate and the incubation medium supernatants were also determined to give a measurement total amount of label incorporated into the tissue/cells. The amount of [^{3}H]-AA released could then be expressed as a percentage of the total amount of label incorporated into the tissue/cells.

To examine the effects of inhibitors on [^{3}H]-AA release responses, the tissue was preincubated for 15 min in pre-warmed and pre-gassed MEM containing the appropriate inhibitor or no drug as appropriate. The medium was then removed and discarded, and the tissue incubated with the appropriate stimulus together with the inhibitor as described above.

Since PLA_2 can be markedly activated by raised Ca^{2+} levels (Irvine, 1982; Rosenthal *et al,* 1989; section 1.3.2.1), we tested the effect of the Ca^{2+} ionophore, ionomycin, as a trial stimulus on [^{3}H]-AA release from pre-labelled anterior pituitary tissue. Figure 2.4 shows the effect of ionomycin (30 µM) treatment of [^{3}H]-AA pre-labelled male rat pituitary pieces on the changes in radioactivity in each solvent fraction. After a 15 minute incubation with ionomycin the radioactivity levels in each solvent fraction obtained from ODS-extraction of the medium were increased (Figure 2.4a). In particular, ionomycin treatment significantly enhanced the levels of radioactivity in fraction D (petroleum ether:$CHCl_3$) ($p < 0.05$, $n = 5$; Mann-Whitney U-test). Additional experiments showed that when [^{3}H]-AA was incubated with anterior pituitary tissue which had been previously inactivated by homogenising in 1 ml MEM, diluted in ethanol to a final concentration of 30% and carried through the extraction procedure, over 83% authentic [^{3}H]-AA was detected in the petroleum

ether:$CHCl_3$ fraction. This observation correlates with those made by Powell (1982) who showed that, whereas the more polar phospholipids were probably passed through the column without being retained, fatty acids, such as AA, and monohydroxy fatty acids were eluted by mixtures of petroleum ether and $CHCl_3$. The radioactivity in fraction E (methyl formate) of the medium extract was also slightly increased following ionomycin treatment. Since methyl formate can elute prostaglandins and thromboxanes from the ODS column, it would seem, therefore, that ionomycin treatment of anterior pituitary also induces an increase in medium AA metabolite levels. The radioactivity levels in fractions F, G and H (80% ethanol, 100% ethanol and distilled H_2O respectively) from the medium were also increased following ionomycin treatment. These fractions are believed to contain moderately polar hydrophilic substances whose exact identity is uncertain (Powell, 1982).

Ionomycin treatment had no significant effect on the levels of radioactivity in the solvent fractions obtained form ODS-extraction of the tissue homogenate (Figure 2.4b). Interestingly, the radioactivity in fraction F (80% ethanol) was decreased in the ionomycin-treated tissue samples. The reason for this decrease in unknown but may simply be a reflection of the relatively small sample size used in this study.

In summary, ionomycin treatment of [^{3}H]-AA pre-labelled male rat anterior pituitary tissue increases medium [^{3}H]-AA levels, as assessed by the levels of radioactivity in the petroleum ether:$CHCl_3$ fraction from ODS extraction. This technique of measuring [^{3}H]-AA release from pre-labelled anterior pituitary tissue is a useful approach to assess the involvement of AA-releasing enzymes, such as PLA_2 or DAG lipase in agonist responses, but must be used with restricted interpretation and in internally controlled comparative experiments. It is unclear as to how measurements made using this assay reflect cytosolic [^{3}H]-AA levels during a stimulus since there is, of course, no information on absolute [^{3}H]-AA concentrations, just relative levels of radioactivity. Mass assay measurements of cellular AA levels

have been described (Currie *et al,* 1992), but such measurements are impossible here due to the low abundance of the cell type relevant to this research project (i.e. gonadotrophs).

To investigate the source of the baseline release of [^{3}H]-AA, pro-oestrous rat anterior pituitary tissue, which had been pre-labelled with [^{3}H]-AA, was incubated for 15 min in MEM containing either no drug (baseline), or the PLA_2 inhibitor quinacrine (50 μM) or the DAG lipase inhibitor, RHC 80267 (200 μM), and the medium [^{3}H]-AA levels were determined (Table 2.1). Neither quinacrine nor RHC 80267 had any significant inhibitory effect on baseline [^{3}H]-AA release suggesting that neither PLA_2 nor DAG lipase plays any significant role in the basal turnover of AA in this tissue. Baseline [^{3}H]-AA release may represent the leak of unesterified fatty acid from the tissue.

Since the main aim of this research project has been to elucidate the targets and actions of the PKC(s) involved in anterior pituitary hormone release, the effects of DAG lipase and PLA_2 inhibitors were examined on phorbol 12,13-dibutyrate (PDBu)-induced [^{3}H]-AA release from pre-labelled pro-oestrous rat anterior pituitary tissue (Table 2.2). In the presence of PDBu (300 nM), [^{3}H]-AA release was significantly increased above baseline levels. When used at a concentration that is known to block PLA_2 activity with minimal side-effects on other cellular processes, quinacrine (50 μM) inhibited PDBu-induced [^{3}H]-AA release to levels that were not significantly different from baseline. However, RHC 80267, at a concentration (80 μM) which has been shown to inhibit the cellular actions of DAG lipase with little side-effect (Sutherland and Amin, 1982), had no significant inhibitory effect on PDBu-induced [^{3}H]-AA release. Within the limits of the pharmacological specificity of these tools, these results suggest that, in pro-oestrous rat anterior pituitary tissue, PKC activation induces [^{3}H]-AA release by a route involving activation of PLA_2, but not DAG lipase.

2.2.6 Cytosolic [^{3}H]-phorbol 12,13-dibutyrate binding studies

The number of PKC molecules in E_2-treated and -untreated pituitary cytosol and their affinity for PDBu were assessed using a [^{3}H]-PDBu binding method described previously (Leach *et al,* 1983, Figure 2.5). Anterior pituitaries were homogenised in 2 vol 50 mM Tris HCl (pH 9.0) containing 1 mM PMSF and 1 mM $MnCl_2$ then centrifuged (100,000 g, 1 h, 4°C). The supernatant was collected and re-centrifuged (120,000 g, 1 h, 4°C) and the supernatant from the second spin was regarded as cytosol and stored at -40°C until use. Cytosol was diluted in assay buffer (50 mM Tris HCl (pH 7.4), 4 mg/ml BSA (essential fatty acid free), 1 mM $CaCl_2$ and 75 mM magnesium acetate) to a concentration which gave total binding of approximately 5 - 10% of total radioactivity present. The assay constituents were 1 mg/ml phosphatidylserine (sodium salt), [^{3}H]-PDBu (0.2 - 50 nM final concentration) and 25 µl of diluted cytosol. Phosphatidylserine vesicles were prepared by dissolving the lipid in chloroform, drying under N_2, reconstituting into assay buffer, vortexing and sonicating. Total binding was measured in the presence of 0.5% DMF whilst non-specific binding was measured in the presence of 20 µM PDBu (with DMF vehicle to 0.5%). After a 30 min incubation (37°C), protein was precipitated at 4°C by adding 100 µl of 12 mg/ml bovine gamma-globulin and 300 µl of 24% polyethyleneglycol 8000 in 50 mM Tris HCl (pH 7.4) (1.8 mg/ml and 11% final concentrations respectively) (4°C, 20 min). The precipitate was pelleted by centrifugation (12,000 g, 5 min, 4°C), the supernatant aspirated off and the radioactivity in each pellet determined by being dissolved overnight in 10 ml scintillation fluid, then liquid scintillation counted for β-radioactivity.

2.2.7 [^{3}H]-N,N-Dimethylstaurosporine binding studies

The method used was based on that described by Gross *et al,* 1990 (Figure 2.6). Tissue was homogenised in buffer containing Tris-HCl (20 mM), EtSH (50 mM), EDTA (2 mM) and PMSF (1 mM), pH 7.5. Tissue homogenate was

centrifuged (12,000g, 4°C, 20 min), the supernatant was removed and respun (12,000g, 4°C, 20 min). The supernatant from the second spin was regarded as cytosol and was used in the [^{3}H]-N,N-dimethylstaurosporine ([^{3}H]-DMS) binding studies. Cytosol was diluted in homogenisation buffer to a concentration which gave total binding of approximately 10% of total radioactivity present. The assay constituents were (final concentrations) Tris-HCl (20 mM pH 8.0), 0.2 μg/ml bovine gamma globulin, 1 mM dithiothreitol, 0.5 nM [^{3}H]-DMS, unlabelled PKC inhibitor and diluted cytosol. After 30 minutes incubation at 4°C, the protein was precipitated by adding 12 mg/ml bovine gamma globulin and 24% polyethylene glycol 8000 (final concentrations of 1.8 mg/ml and 11% respectively) (4°C, 20 min). The precipitate was pelleted by centrifugation (12,000 g, 5 min, 4°C), the supernatant aspirated off and the radioactivity in each pellet counted.

Total binding (TB) was measured in the absence of unlabelled PKC inhibitor and in the presence of 0.5% DMF. Non-specific binding (NSB, which was approximately 25% of TB) was determined in the presence of 3 μM staurosporine in a final DMF concentration of 0.5%.

2.2.8 Protein assay

Protein content in anterior pituitary cytosol, and in DEAE cellulose-purified cytosol was measured using a Pierce protein assay kit (Pierce, Chester, UK). This method uses an assay reagent based on the Bradford method (1976) consisting of Coomassie Blue G-250, phosphoric acid, methanol, water and solubilizing agents. When Coomassie Blue binds to proteins in an acidic solution, an absorbance shift from 465 to 595 nm occurs. Protein standard was BSA (essential fatty acid free) diluted in the appropriate assay medium. The protein concentrations for the unknown samples were determined against the standard curve, which was consistently linear with the standard protein concentration used.

2.2.9 Data analysis

All values are expressed as mean ± standard error of the mean (SEM) from data obtained from a number (n) of independent, individual determinations which were performed either on the same day or on two consecutive days. Statistical analyses were carried out using the Mann-Whitney U-test unless otherwise stated. The time-course data in Chapter 5 were analysed by both 2-way and 1-way analysis of variance, followed by Duncan's New Multiple Range test on statistically different groups. The concentration of drug which could produce 50% of the maximal response (EC_{50} value) and the concentration of inhibitors which could inhibit 50% of a stimulus-evoked response (IC_{50} value) were assessed by fitting the data with a non-linear, error-weighted, iterative curve-fitting programme, P.fit (Biosoft, Cambridge, UK) and represented as means ± SEM.

FIGURE 2.1

Flow diagram of the method used to measure anterior pituitary hormone release from rat anterior pituitary pieces *in vitro*

Figure 2.1

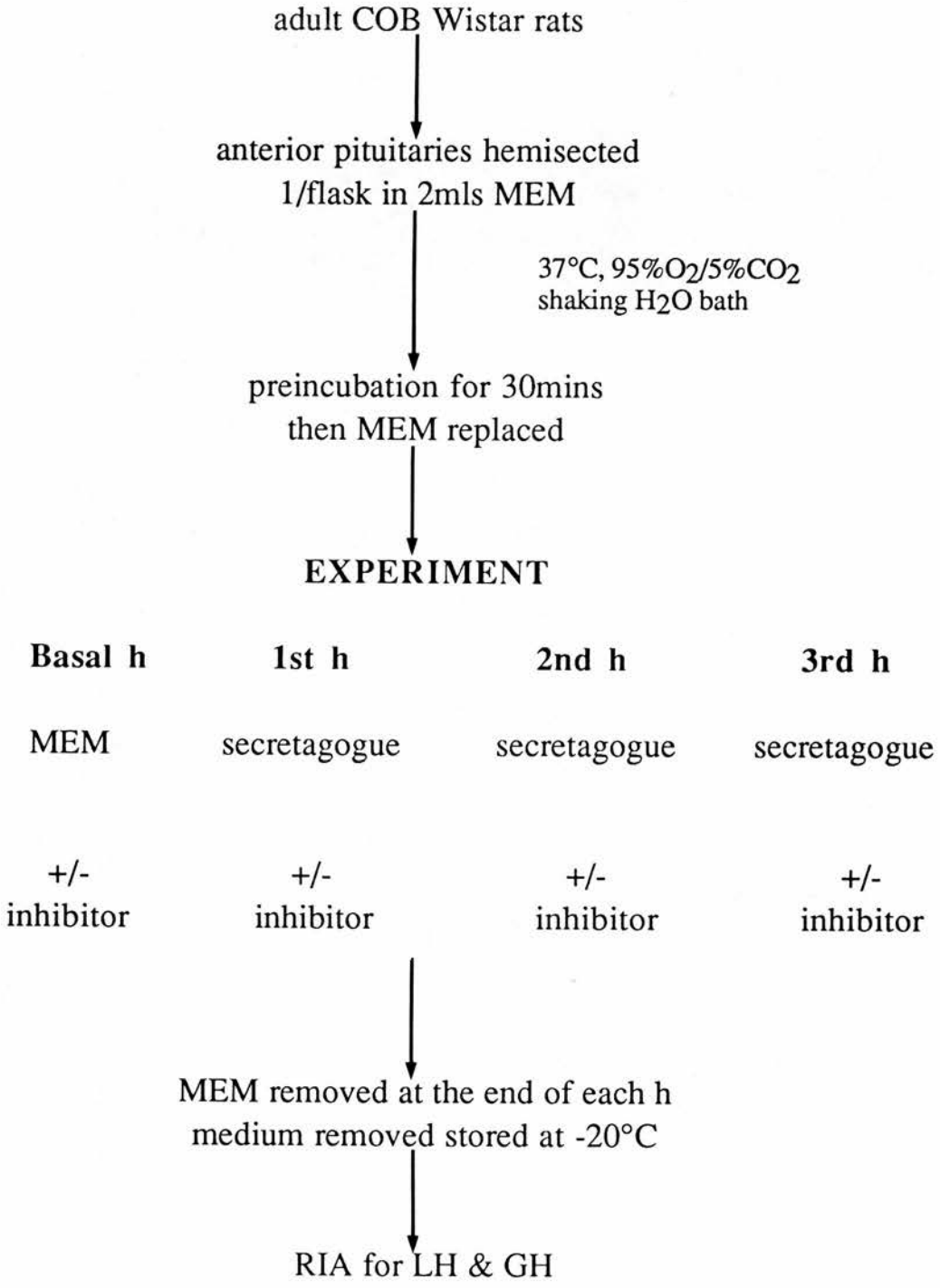

adult COB Wistar rats
anterior pituitaries hemisected
1/flask in 2mls MEM
37°C, 95%O2/5%CO2
shaking H2O bath
preincubation for 30mins
then MEM replaced
EXPERIMENT
Basal h
1st h
2nd h
3rd h
MEM
secretagogue
secretagogue
secretagogue
+/-
inhibitor
+/-
inhibitor
+/-
inhibitor
+/-
inhibitor
MEM removed at the end of each h
medium removed stored at -20°C
RIA for LH & GH

FIGURE 2.2

Flow diagram of the methodology used to determine cytosolic protein kinase C activity

Figure 2.2

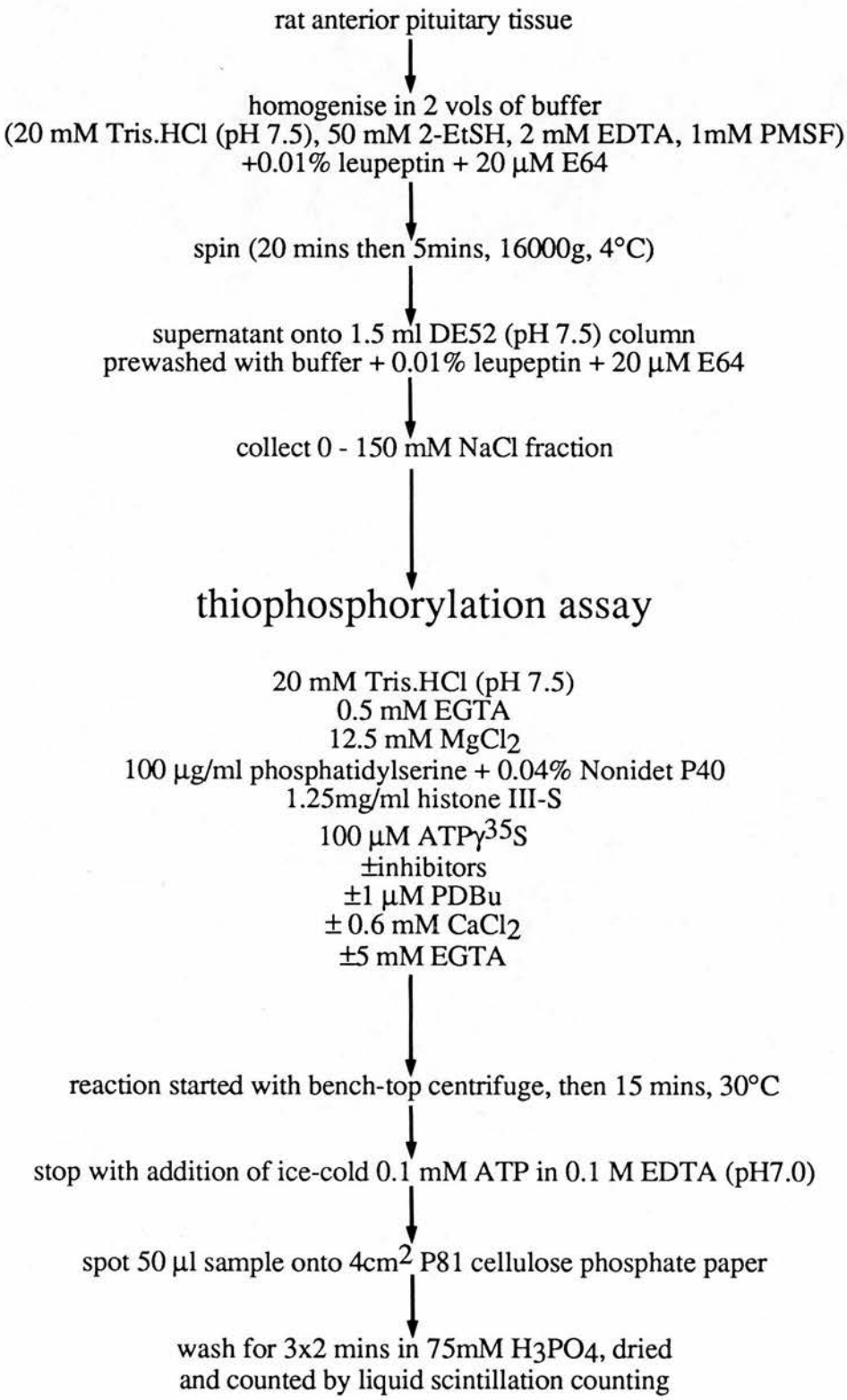

rat anterior pituitary tissue
homogenise in 2 vols of buffer
(20 mM Tris.HCl (pH 7.5), 50 mM 2-EtSH, 2 mM EDTA, 1mM PMSF)
+0.01% leupeptin + 20 µM E64
spin (20 mins then 5mins, 16000g, 4°C)
supernatant onto 1.5 ml DE52 (pH 7.5) column
prewashed with buffer + 0.01% leupeptin + 20 µM E64
collect 0 - 150 mM NaCl fraction
thiophosphorylation assay
20 mM Tris.HCl (pH 7.5)
0.5 mM EGTA
12.5 mM MgCl2
100 µg/ml phosphatidylserine + 0.04% Nonidet P40
1.25mg/ml histone III-S
100 µM ATPγ35S
±inhibitors
±1 µM PDBu
± 0.6 mM CaCl2
±5 mM EGTA
reaction started with bench-top centrifuge, then 15 mins, 30°C
stop with addition of ice-cold 0.1 mM ATP in 0.1 M EDTA (pH7.0)
spot 50 µl sample onto 4cm2 P81 cellulose phosphate paper
wash for 3x2 mins in 75mM H3PO4, dried
and counted by liquid scintillation counting

FIGURE 2.3

Flow diagram of the methods used to measure [^{3}H]-arachidonic acid release from rat anterior pituitary pieces and dispersed anterior pituitary cells

Figure 2.3

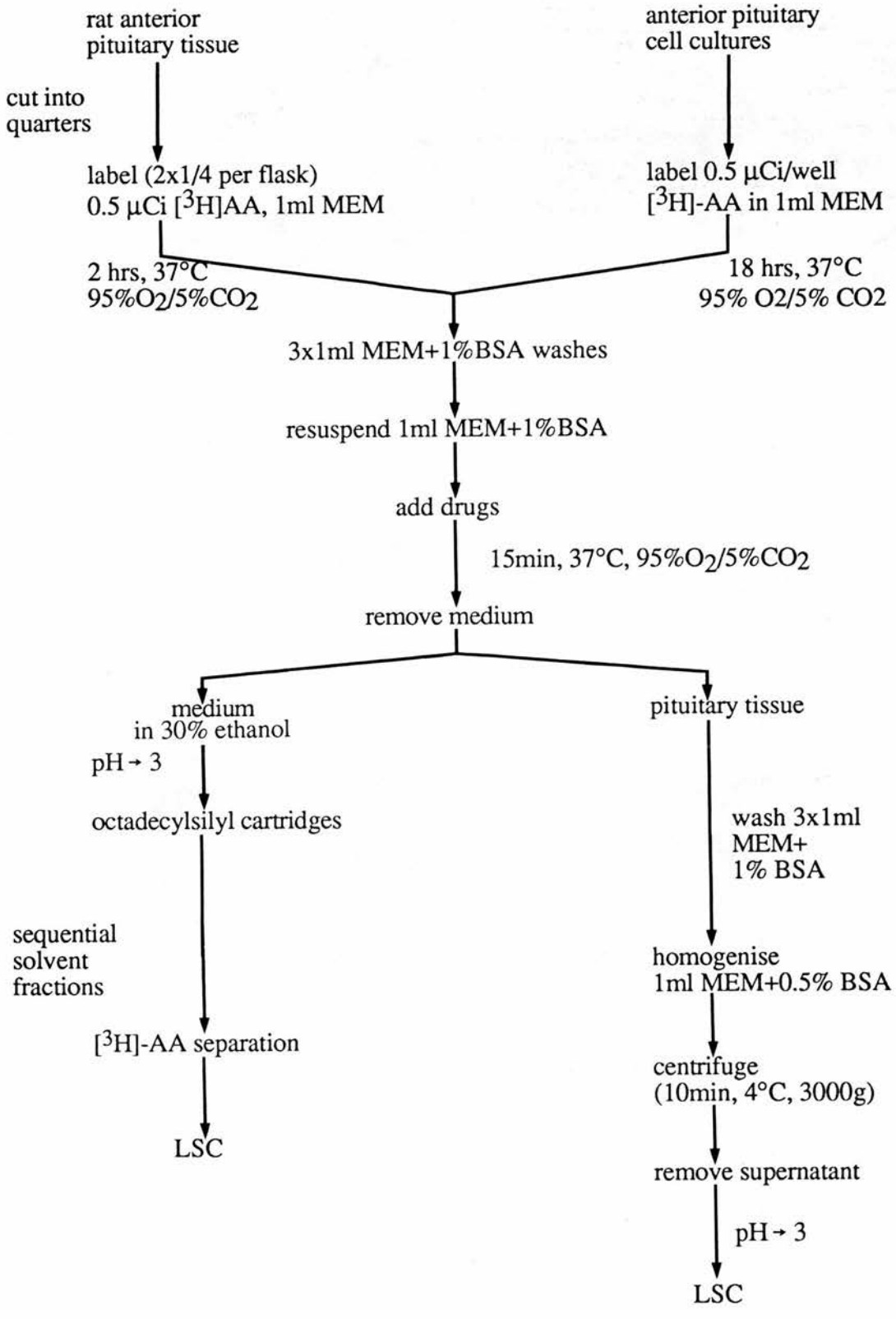

rat anterior pituitary tissue
cut into quarters
label (2x1/4 per flask) 0.5 µCi [3H]AA, 1ml MEM
2 hrs, 37°C 95%O2/5%CO2
anterior pituitary cell cultures
label 0.5 µCi/well [3H]-AA in 1ml MEM
18 hrs, 37°C 95% O2/5% CO2
3x1ml MEM+1%BSA washes
resuspend 1ml MEM+1%BSA
add drugs
15min, 37°C, 95%O2/5%CO2
remove medium
medium in 30% ethanol
pH → 3
octadecylsilyl cartridges
sequential solvent fractions
[3H]-AA separation
LSC
pituitary tissue
wash 3x1ml MEM+ 1% BSA
homogenise 1ml MEM+0.5% BSA
centrifuge (10min, 4°C, 3000g)
remove supernatant
pH → 3
LSC

FIGURE 2.4

Levels of radioactivity in solvent fractions from (a) medium and (b) tissue from control and ionomycin-treated male rat anterior pituitary pieces

Male rat tissue was incubated in medium containing either no drug (baseline) or ionomycin (30 μM) for 15 min. The radioactivity in the solvent fractions of the medium (a) and tissue (b) from each treatment was determined. The fractions are as follows; A: distilled H_2O; B: 30% ethanol; C: petroleum ether; D: petroleum ether: $CHCl_3$ (1:1); E: methyl formate; F: 80% ethanol; G: 100% ethanol; H: distilled H_2O. The statistical significance of the effects of ionomycin was determined (*$p \leq 0.05$, Mann-Whitney U-test). Values are means ± SEM for 4 - 5 determinations.

Figure 2.4

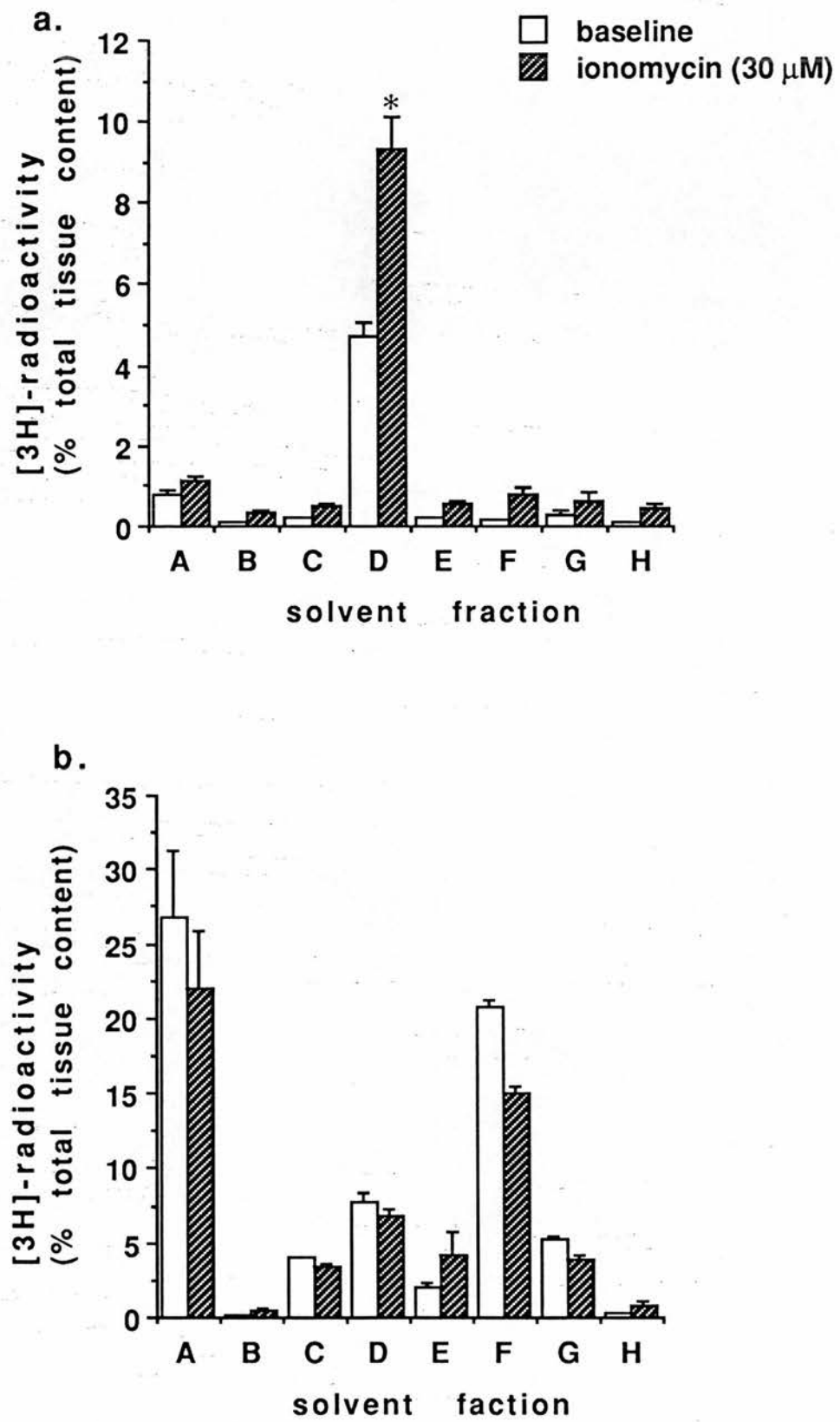

a.
baseline
ionomycin (30 μM)
[3H]-radioactivity
(% total tissue content)
12
10
8
6
4
2
0
*
A
B
C
D
E
F
G
H
solvent fraction
b.
[3H]-radioactivity
(% total tissue content)
35
30
25
20
15
10
5
0
A
B
C
D
E
F
G
H
solvent faction

FIGURE 2.5

Flow diagram of the method used to measure [^{3}H]-phorbol 12,13-dibutyrate binding in these studies

Figure 2.5

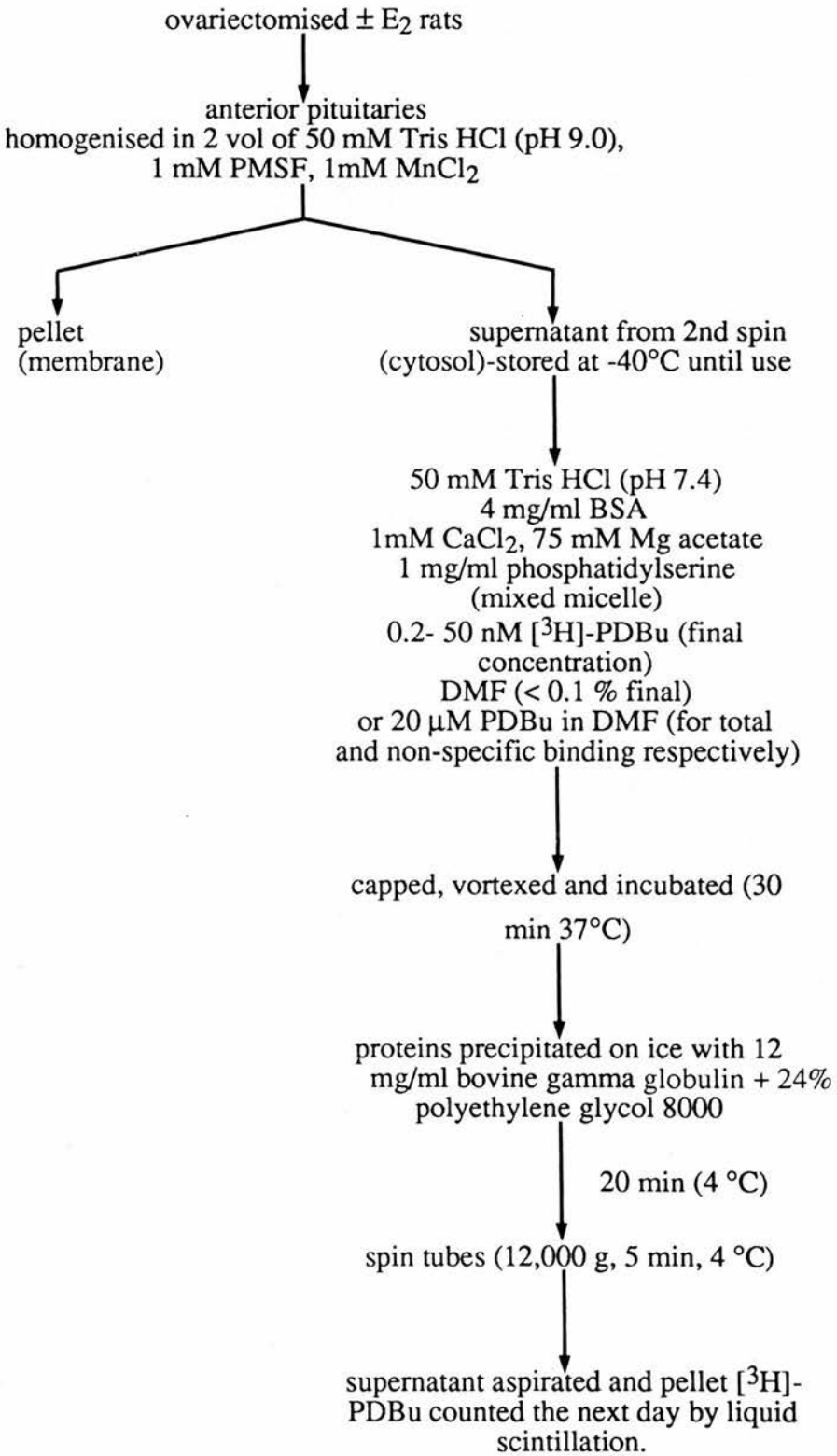

ovariectomised ± E_2 rats
anterior pituitaries
homogenised in 2 vol of 50 mM Tris HCl (pH 9.0),
1 mM PMSF, 1mM $MnCl_2$
pellet
(membrane)
supernatant from 2nd spin
(cytosol)-stored at -40°C until use
50 mM Tris HCl (pH 7.4)
4 mg/ml BSA
1mM $CaCl_2$, 75 mM Mg acetate
1 mg/ml phosphatidylserine
(mixed micelle)
0.2- 50 nM [^{3}H]-PDBu (final concentration)
DMF (< 0.1 % final)
or 20 µM PDBu in DMF (for total and non-specific binding respectively)
capped, vortexed and incubated (30 min 37°C)
proteins precipitated on ice with 12 mg/ml bovine gamma globulin + 24% polyethylene glycol 8000
20 min (4 °C)
spin tubes (12,000 g, 5 min, 4 °C)
supernatant aspirated and pellet [^{3}H]-PDBu counted the next day by liquid scintillation.

FIGURE 2.6

Flow diagram of the [^{3}H]-N,N-dimethylstaurosporine binding methodology used in these studies

Figure 2.6

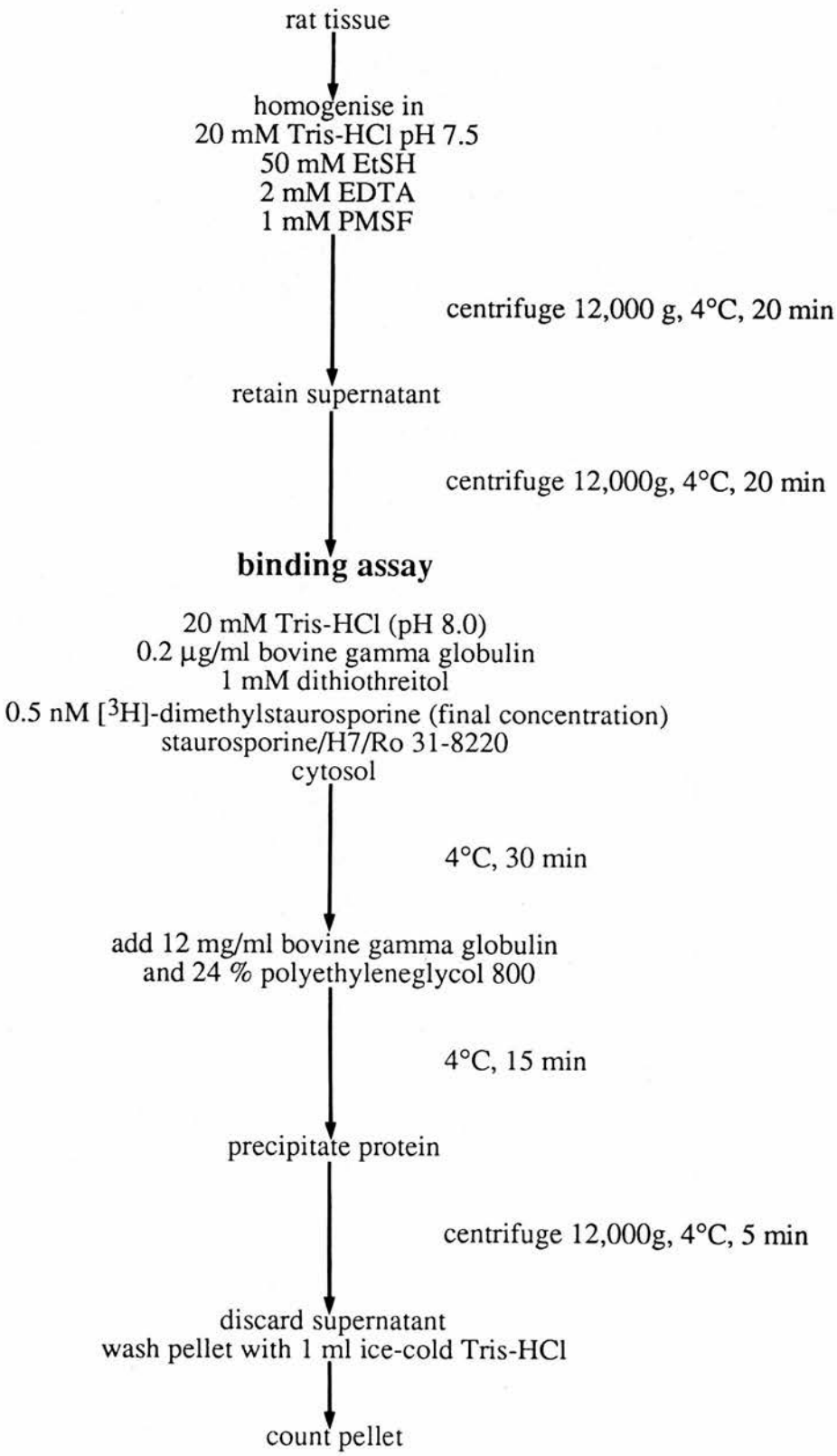

rat tissue
homogenise in
20 mM Tris-HCl pH 7.5
50 mM EtSH
2 mM EDTA
1 mM PMSF
centrifuge 12,000 g, 4°C, 20 min
retain supernatant
centrifuge 12,000g, 4°C, 20 min
binding assay
20 mM Tris-HCl (pH 8.0)
0.2 µg/ml bovine gamma globulin
1 mM dithiothreitol
0.5 nM [3H]-dimethylstaurosporine (final concentration)
staurosporine/H7/Ro 31-8220
cytosol
4°C, 30 min
add 12 mg/ml bovine gamma globulin
and 24 % polyethyleneglycol 800
4°C, 15 min
precipitate protein
centrifuge 12,000g, 4°C, 5 min
discard supernatant
wash pellet with 1 ml ice-cold Tris-HCl
count pellet

TABLE 2.1

The effect of quinacrine and RHC 80267 on baseline [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pre-labelled pro-oestrous rat anterior pituitary pieces

Anterior pituitary pieces, which had been pre-labelled with [^{3}H]-AA and extensively washed, were incubated for 15 min in medium containing either no drug (baseline), quinacrine (50 μM) or RHC 80267 (200 μM). Medium [^{3}H]-AA levels were determined as described in section 2.2.5. Data are means ± SEM and the number of determinations are shown in parentheses.

	[^{3}H]-AA (% of total label incorporated)
baseline	0.69 ± 0.07 (8)
quinacrine	0.61 ± 0.02 (4)
RHC 80267	0.69 ± 0.08 (4)

TABLE 2.2

The effect of quinacrine and RHC 80267 on phorbol 12,13-dibutyrate (PDBu)-induced [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pre-labelled pro-oestrous rat anterior pituitary pieces

[^{3}H]-Arachidonic acid-labelled pairs of anterior pituitary quarters were pre-incubated for 15 minutes in medium only, or with quinacrine (50 μM) or RHC 80267 (80 μM), the medium was discarded and replaced with fresh medium containing either no drug (basal) or PDBu (300 nM), or PDBu and either quinacrine or RHC 80267. After a second 15 min incubation, the medium was removed and the [^{3}H]-AA released was extracted. The statistical significance of the effects of quinacrine and RHC 80267 on PDBu-induced [^{3}H]-AA release was determined (*$p < 0.05$, Mann-Whitney U-test). Values shown are the means ± SEM and the number of determinations are shown in parentheses.

	[^{3}H]-AA release (% of total label incorporated)
basal	1.59 ± 0.07 (10)
PDBu	2.73 ± 0.16 (8)
PDBu + quinacrine	*1.65 ± 0.02 (5)
PDBu + RHC 80267	2.53 ± 0.22 (4)

CHAPTER 3

EVIDENCE FOR A ROLE OF PROTEIN KINASE C IN THE MECHANISM OF GONADOTROPHIN RELEASE FROM RAT ANTERIOR PITUITARY TISSUE

3.1 INTRODUCTION

Although LHRH receptor activation in rat anterior pituitary gonadotrophs is associated with rapid metabolism of phosphoinositides (Snyder and Bleasdale, 1982; Raymond *et al,* 1984) and production of DAG (Andrews and Conn, 1986), which can activate PKC, the exact involvement of PKC in LHRH responses is controversial (see Conn, 1989 for review). Luteinizing hormone-releasing hormone can induce PKC translocation to the cell membrane (Hirota *et al,* 1985; Naor *et al,* 1985; McArdle and Conn, 1986) and PKC activators induce gonadotrophin release, *in vitro* (Smith and Vale, 1980; Conn *et al,* 1985; Nigro-Villar and Lapetina, 1985; Turgeon and Waring, 1986), consistent with a role for PKC in gonadoptrophin release. Some studies have shown that LHRH-induced LH release can be prevented by PKC inhibitors (Hirota *et al,* 1985; Chang *et al,* 1987b), whilst others have reported that LHRH responses are unaffected by these drugs (Johnson *et al,* 1988; Beggs and Millar, 1989). Investigations into the effect of PKC down-regulation on secretory responses to LHRH in cultured gonadotrophs have also produced conflicting results; some have reported PKC down-regulation attenuates responses to LHRH (Stojilkivic *et al,* 1988b, 1991), whereas others suggest that LHRH-induced LH release is unaffected (McArdle and Conn, 1986; McArdle *et al,* 1987). Clearly, additional studies are required to examine a possible role for PKC in responses to LHRH.

Evidence suggests that the gonadotrophin release response to LHRH is probably under the influence of several different, cellular factors. For example, the biphasic pattern of LHRH-induced gonadotrophin release observed in cultured cells (Borges *et al,* 1983) correlates with changes in the requirement of the response for Ca^{2+} from intracellular and extracellular sources (Bourne and Baldwin, 1980; Shangold *et al,* 1988; Smith *et al,* 1987;Tasaka *et al,* 1988; see Davidson *et al,* 1991 for review). In addition, acute LHRH responses measured *in vitro* are independent of

protein synthesis whereas later responses to LHRH, including the LHRH priming response, (Aiyer *et al,* 1974a), can be prevented by protein synthesis blockers (Edwardson and Gilbert, 1976; Pickering and Fink, 1976; Bourne and Baldwin, 1980). Since the release of gonadotrophin induced by LHRH may be under the influence of different factors at any one particular time, it is likely that PKC activation is only one of a number of intracellular changes that occur following LHRH receptor activation and will, therefore, have a select role in the temporal pattern of gonadotrophin release.

In an attempt to clarify the role of PKC activation in LHRH receptor signalling, the effects of a number of putative inhibitors of this kinase were examined on initial responses to LHRH and on the later LHRH priming effect which is observed in pro-oestrous rat anterior pituitary pieces. There is now evidence which suggests that distinct PKC isoforms show different sensitivities to various PKC activators and inhibitors (Evans *et al,* 1991; Ryves *et al,* 1991). Therefore, to investigate the possibility that pharmacologically distinct PKCs are involved in LHRH priming, LH release, and, as a contrast, growth hormone (GH) release, the effects of PKC inhibitors were also examined on hormone release induced by various pharmacological activators of PKC.

3.2 SPECIFIC METHODOLOGY

Stimulus-induced hormone release was measured from anterior pituitary pieces as described in section 2.2.3. Pro-oestrous female rat hemipituitaries were incubated for an initial hour (basal h) in medium only, followed by consecutive hours (1st, 2nd, 3rd h) in the presence of either LHRH or a PKC activator (phorbol 12,13-dibutyrate (PDBu), 1,2-dioctanoyl-*sn*-glycerol (DOG) or mezerein) or a Ca^{2+} ionophore (ionomycin). To examine the effects of PKC inhibitors (staurosporine, K252a, H7, Ro 31-8220) on hormone release, the appropriate inhibitor was included in the basal h incubation and in the 1st h, 2nd h, 3rd h, together with the secretagogue.

To control for the effects of each PKC inhibitor on baseline hormone release, some tissue samples were incubated for consecutive hourly periods in the presence of inhibitor only. Phorbol 12,13-dibutyrate, mezerein, DOG, ionomycin, staurosporine, K252a and Ro 31-8220 were all made up as stock solutions in dimethylformamide (DMF) and kept in the dark, on ice, before final dilution in MEM for experiments. A fresh stock of DOG was made for every experiment. The maximum concentration of DMF used was 0.5% v/v and this was shown in control experiments to have no effect. H7 and LHRH were made up as stock solutions in distilled H_2O.

To examine the role of PKC in LHRH priming in more detail, a number of different experiments were carried out. In a first experiment, staurosporine was included in the basal h and in the 1st h together with LHRH. The tissue was then washed in MEM only, in a shaking water bath (under 95% O_2/5% CO_2, at 37°C) for 2 consecutive 5 minute periods, to remove the staurosporine. At the end of each 5 min wash, the medium was discarded and replaced with fresh MEM. Following staurosporine washout, tissue was incubated for a final h (2nd h) with LHRH only. Control treatments were incubated with LHRH, as above, but in the absence of staurosporine, followed by an identical washing procedure and a 2nd h incubation with LHRH. The effectiveness of the staurosporine washout procedure was tested by incubating tissue with the radiolabelled derivative of staurosporine, [^{3}H]-N, N-dimethylstaurosporine ([^{3}H]-DMS), over 2 consecutive hours. The medium was then removed, and the tissue washed as described above, but each wash was retained. After washing, the tissue was homogenised in 1 ml of fresh MEM and the radioactivity in the tissue homogenate and the two 5 ml washes was measured by liquid scintillation counting. The tissue concentration of [^{3}H]-DMS remaining after washout was calculated as a % of the total radioactivity in the tissue prior to washout.

In a second experiment, the effect of staurosporine on LHRH responses in pre-primed tissue was examined. The tissue was incubated for one hour in medium only (basal h), followed by a 1st h incubation in the presence of LHRH (1 nM),

during which, LHRH priming occurs. The tissue was then incubated either with LHRH only (control) for 2 further consecutive hours (2nd, 3rd), or with LHRH and staurosporine (300 nM).

In a third experiment, the effect of staurosporine on ionomycin-induced gonadotrophin release from preprimed tissue was examined. Tissue was either incubated in medium only (basal h), followed by LHRH (1 nM) for a 1st h then with ionomycin (30 μM) for a 2nd h or together with staurosporine throughout each of these hourly incubations.

All statistical analyses were carried out using the Mann-Whitney U-test. The standards used were NIADDK-rat FSH-RP2 for all FSH data, NIADDK-rat-GH-RP-2 for all GH data, NIH-LH-S18 for LH data in Figures 3.2a, 3.2b, 3.5, 3.6, 3.8, 3.9a, and NIADDK-rat LH-RP2 for the remaining figures and tables.

3.3 RESULTS

The effect of PKC inhibitors on initial LHRH-induced gonadotrophin release and LHRH priming in pro-oestrous rat anterior pituitary tissue

Figure 3.1 shows the effect of consecutive hourly incubations of LHRH (1 nM) on (a) LH and (b) FSH release from pro-oestrous rat anterior pituitary tissue. During the 1st h incubation with LHRH, LH release was significantly increased from a basal h level of 6.3 ± 1.5 μg/l (n = 6) to 29.9 ± 8.1 μg/l (n = 6). With a 2nd h incubation with LHRH, LH release was greatly enhanced with respect to that measured during the 1st h (162.0 ± 20.4 μg/l). This enhanced LHRH-induced LH release occurs as a result of LHRH priming (Aiyer *et al,* 1974). Similarly, LHRH treatment induced a significant increase in initial acute FSH release followed by an further enhanced FSH release response in the 2nd h.

To investigate the role of PKC in LHRH responses, the PKC inhibitor, staurosporine (300 nM), was included throughout each hourly incubation (Figure 3.1). In the presence of staurosporine (hatched bars), neither basal h nor 1st h LHRH-

induced LH or FSH release were significantly different from control levels (open bars). However 2nd h LHRH-induced LH and FSH were both significantly inhibited by staurosporine ($p < 0.05$). Interestingly, at staurosporine concentrations of 300 nM and above, 2nd h LH (Figure 3.2a) and FSH release (Figure 3.3a) were only ever decreased to levels seen in the 1st h with LHRH, but not to basal h levels. Thus, it appears that staurosporine blocks the enhanced secretory response to LHRH, that occurs as a consequence of LHRH priming, without affecting acute, 1st h LHRH-induced release. Similarly, the staurosporine congener, K252a, inhibited the 2nd h, primed LH and FSH response without altering initial LHRH-induced gonadotrophin release (Figures 3.2b and 3.3b).

Recently, a staurosporine derivative, Ro 31-8220, has been developed with considerably greater selectivity for PKC (Davis *et al,* 1989) than staurosporine itself. Similar to the effects of staurosporine, Ro 31-8220 was found to significantly inhibit the 2nd h of LHRH-induced LH (Figure 3.2c) without altering either basal or 1st h LHRH-induced gonadotrophin release. The inhibitory effects of Ro 31-8220 on 2nd h LHRH-induced LH release further suggest that activation of PKC (or a closely related kinase), is required for LHRH priming.

It has been reported that another PKC inhibitor, H7, at a concentration of 10 μM, is unable to prevent LHRH priming (Johnson *et al,* 1988). Since staurosporine and H7 are both thought to inhibit kinase activity by interacting with, or near to, the ATP binding site of the enzyme, the dissociated effects of these compounds on priming may be unexpected. However, it has been suggested that certain actions of PKC may be less sensitive to blockade by H7 than has been generally reported (Watson *et al,* 1988; Nakadate *et al,* 1989; MacEwan and Mitchell, 1991). To investigate the possiblity that LHRH priming involves a PKC which is relatively H7-resistant, the effect of H7 on LHRH responses was examined in more detail (Figures 3.2d and 3.3c). Consistent with a previous report (Johnson *et al,* 1988), 10 μM H7 had no significant effect on either 1st h or 2nd h LHRH-induced LH

or FSH release. However, at H7 concentrations of 100 μM and above, 2nd h LHRH-induced LH and FSH release were significantly inhibited ($p < 0.05$). Neither baseline nor 1st h LH release was affected by H7 (10 - 300 μM).

Evidence that PKC activation is required for the induction of LHRH priming, but not for LHRH-induced gonadotrophin release

Since staurosporine, K252a, Ro 31-8220 and H7 reduced 2nd h LHRH-induced gonadotrophin release to 1st h levels only, PKC activation may be essential for enhanced pituitary responsiveness to LHRH only, but not for initial responses to LHRH. It is possible that PKC activation is required either for the induction of LHRH priming, or for the mechanism that directly mediates an increased rate of hormone release which is needed to express priming. To distinguish between these possible roles for PKC in LHRH responses, the mode of involvement of PKC in LHRH priming was examined in a series of experiments (Figure 3.4). Since there was no discernible difference between the effects of PKC inhibitors on LH and FSH release (see above), in these remaining experiments, LH release only was determined.

In a first experiment, after a basal h incubation with staurosporine (300 nM), the inhibitor was included throughout the 1st h of incubation, together with LHRH (1 nM) (i.e. that hour during which priming occurs). The tissue was then washed extensively to remove the staurosporine, and incubated for a 2nd h in the presence of LHRH only. In this experiment, staurosporine did not alter either basal h or initial 1st h LHRH-induced LH release (Figure 3.4a), similar to the effects of staurosporine shown previously (see Figures 3.1 and 3.2). Although staurosporine was not present in the 2nd h of LHRH incubation, LHRH-induced LH release during this hour was still significantly inhibited ($p < 0.05$). When tissue was incubated for 2 consecutive hours with [^{3}H]-N, N-dimethylstaurosporine ([^{3}H]-DMS), and subjected to an identical washing procedure, the tissue concentration of [^{3}H]-DMS remaining was reduced to 8 ± 2% ($n = 4$) of the initial level. Furthermore, to test that PKC was

still active following washout, tissue was incubated with staurosporine for 2 h, washed and the PDBu facilitation of K^+-induced $^{45}Ca^{2+}$ influx was measured as described by MacEwan and Mitchell (1991). Following staurosporine washout, PDBu enhancement of K^+-induced $^{45}Ca^{2+}$ influx was not significantly different from that in control tissue (108 ± 10% of the facilitation measured in vehicle-incubated controls). Thus, anterior pituitary PKC(s) are still active after staurosporine treatment and subsequent washout.

In a second experiment, the effect of staurosporine on LHRH responses in pre-primed tissue was tested (Figure 3.4b). In this experiment, tissue was primed by incubating with LHRH (1 nM) for a 1st h, then staurosporine (300 nM) was included during the 2nd and 3rd h incubations together with LHRH. Staurosporine had no significant inhibitory effect on LHRH-induced LH or FSH release from pre-primed anterior pituitary tissue, even over a period of two hours which would allow for the expression of any possible slow-developing inhibitory effect.

Finally, to confirm that staurosporine has no inhibitory action on the general secretory apparatus of the gonadotroph, the effect of staurosporine on ionomycin-induced gonadotrophin release was examined (Table 3.1). Luteinizing hormone release was significantly increased over all hours of incubation with ionomycin (30 μM), but did not exhibit a pattern of release typical of a 'primed' response. Ionomycin-induced LH release was not significantly blocked by staurosporine at any hour of incubation with the Ca^{2+} ionophore (1st h, 2nd h), indicating that staurosporine has no inhibitory action on the general, Ca^{2+}-triggered secretory mechanisms in the gonadotroph. When pituitary tissue was incubated with LHRH (1 nM) during the 1st h followed by ionomycin (30 μM) in the 2nd h, the 2nd h ionomycin-induced LH release response was enhanced with respect to 1st h LHRH-induced LH release. These results are consistent with previous observations which show that, although induction of priming is specific to LHRH, an increased gonadotrophin release from previously primed tissue can be elicited by a non-specific

secretagogue, such as ionomycin (Pickering and Fink, 1979). When staurosporine was included throughout each incubation, 1st h LHRH-induced LH release was unaffected but the subsequent 2nd h ionomycin-induced LH release was significantly blocked. This staurosporine block of the 2nd h ionomycin response following LHRH treatment suggests that PKC activation must be important for the induction of LHRH priming, but not for LH release, since LH release induced over consecutive hours with ionomycin only is unaffected by staurosporine.

The effect of PKC activators on luteinizing hormone and growth hormone release from pro-oestrous rat anterior pituitary pieces

When rat hemipituitary pieces were incubated for consecutive hours together with PDBu (100 nM) both LH and GH release were significantly increased, but with very different time courses (Figure 3.5). Release of GH was maximal in the 1st h of phorbol treatment, having increased from a basal h level of 370 ± 89 μg/l (n = 5) to 766 ± 95 μg/l (n = 5) in the 1st h. In contrast, LH release measured during 1st h of PDBu incubation was not significantly different from basal hour levels of LH release. However, LH release was significantly increased ($p < 0.05$) during the 2nd h of PDBu incubation in comparison to basal h levels, and increased further during the 3rd h of PDBu incubation.

Mezerein induced both LH and GH release with a similar temporal pattern to that observed using PDBu. Increasing the concentrations of either PDBu or mezerein was accompanied by a concentration-dependent increase in the magnitude of both LH (Figure 3.6a) and GH release (Figure 3.6b). Growth hormone release was induced by mezerein and PDBu with similar potency. Mezerein was equally effective at releasing both LH (EC_{50} = 152.2 ± 69.8 nM) and GH (EC_{50} = 74.3 ± 62.0 nM). However, PDBu was less effective than mezerein on LH release suggesting that the PKC(s) involved in this process may less susceptible to activation by PDBu.

During the 2nd and 3rd, but not the 1st, consecutive hours of DOG (200 μM) incubation, there was a small, but significant, increase of LH release above baseline levels from pro-oestrous rat anterior pituitary tissue (Figure 3.7a). However, DOG was much less effective at inducing LH release than either PDBu or mezerein. In contrast to the effects on LH release, DOG was unable to induce significant amounts of GH release during any hour of incubation with the diglyceride (Figure 3.7b).

The effect of PKC inhibitors on PKC-activator-induced luteinizing hormone and growth hormone release

The PKC inhibitor staurosporine, caused a concentration-dependent inhibition of 100 nM PDBu-induced LH and GH release with similar potency (IC_{50} = 44.0 ± 17.2 nM and 74.3 ± 62 nM for LH and GH respectively) (Figure 3.8). At staurosporine concentrations of 100 nM and above, both LH and GH release were blocked to levels not significantly different from levels of hormone release measured in the absence of a PDBu stimulus.

Luteinizing hormone release induced by 100 nM PDBu was significantly inhibited by another PKC inhibitor, H7, at concentrations of 3 μM and above and with an IC_{50} value of 1.7 ± 1.5 μM (Figure 3.9a). In contrast, 100 nM PDBu-induced GH release was unaffected by H7, even at concentrations of 30 - 300 μM which caused over 80% inhibition of LH release induced by 100 nM PDBu. However, when the effect of H7 (30 μM) was examined on LH release measured over a range of PDBu concentrations (Figure 3.10), it was clear that an H7-resistant component of this response could also be detected, especially at higher levels of phorbol ester. That is, LH release induced by 30 nM PDBu was completely inhibited by 30 μM H7, whereas, approximately 20% of LH release measured at 1 μM PDBu was insensitive to H7 (30 μM). Similarly, mezerein-induced LH release also consisted of H7-sensitive and -resistant components (Figure 3.9b). Approximately 40% of the LH

secretory response to mezerein was inhibited by H7 at concentrations as low as 1 μM. However, further inhibition of this response was not seen until H7 concentrations of 30 μM and above. Furthermore, at H7 concentrations as high as 100 μM, mezerein-induced LH release was not completely inhibited, with 20% of the response remaining. Mezerein (300 nM) -induced GH release was completely insensitive to inhibition by H7 at concentrations as high as 100 μM (Figure 3.9b). Both mezerein-induced LH and GH release, however, were readily blocked by staurosporine (300 nM) to levels that were not significantly different from baseline levels of release (Table 3.2).

Although DOG (200 μM)-induced LH release was readily inhibited by staurosporine (300 nM), to levels that were not significantly different from baseline, this response was entirely resistant to inhibition by 30 μM H7 (see Table 3.3). Therefore, DOG may be a selective activator of at least some of the H7-resistant PKC(s) which can elicit LH release.

Neither H7 (30 μM) nor staurosporine (2 μM) K252a (10 μM), nor Ro 31-8220 (30 μM) had any effect on baseline LH, FSH and GH release from anterior pituitary tissue when added alone to the incubation medium over a period of 3 consecutive hours .

The effect of PKC inhibitors on PDBu-induced anterior pituitary PKC activity

The effects of H7 and staurosporine were further examined on anterior pituitary PKC activity, measured in a mixed micelle assay. Calcium-independent cytosolic PKC activity was stimulated by PDBu with an EC_{50} of 916 ± 150 nM and the additional activity evoked in the presence of Ca^{2+} had an EC_{50} for PDBu of 261 ± 86 nM. All activity was entirely dependent on the presence of phosphatidylserine. Calcium-dependent activity induced by PDBu (1 μM) was potently inhibited by staurosporine and H7 (Table 3.4) with IC_{50} values similar to those found for PKC activity measured in a range of other peripheral and central tissues. For example,

Ca^{2+}-dependent cytosolic PKC activity from rat midbrain was inhibited by staurosporine and H7 with IC_{50} values of 100 ± 40 nM and 34 ± 5 μM respectively. In contrast, Ca^{2+}-independent anterior pituitary PKC activity was distinctly less sensitive to H7 than the Ca^{2+}-dependent activity measured in the same experiment (Table 3.4). However, in experiments using rat midbrain, Ca^{2+}-independent activity had the expected sensitivity to H7 and staurosporine (IC_{50} = 27 ± 9 μM and 120 ± 6 nM respectively). The Ca^{2+}-independent activity in anterior pituitary, however, showed the expected potency of block by staurosporine (Table 3.4).

3.4 DISCUSSION

The results presented in this chapter demonstrate that activation of PKC (or a closely related kinase) is required for the induction of LHRH priming, but not for initial LHRH-induced gonadotrophin release from pro-oestrous rat anterior pituitary tissue *in vitro*. These results also provide evidence for the existence of several pharmacologically distinguishable PKC forms in anterior pituitary cells and suggest that one or more of these forms have distinct roles in the control of LHRH responses and in PKC activator-induced LH or GH release.

Several lines of evidence presented here, together with observations made by other workers, indicate that the facilitation of gonadotrophin release which is brought about by LHRH priming is dependent upon the activation of a PKC-like kinase and not another known species of protein kinase. Firstly, the LHRH priming response was readily inhibited by staurosporine at concentrations (IC_{50} = 26.3 ± 7.0 nM) typical for inhibition of other PKC-mediated cellular responses (Davis *et al,* 1989; Dunn and Rang, 1990), including the block of 100 nM PDBu-induced LH release (IC_{50} = 44.0 ± 17.2 nM) reported here (Figure 3.8), and for partially- or extensively-purified PKC activity measured in cell free assays (Table 3.4) (Nakadate *et al,* 1988). The potency ratio for the inhibitory effects of staurosporine and its less active congener, K252a, on LHRH priming (62- and 111-fold for FSH and LH

respectively) is of a similar order to that reported for their inhibition of PKC in cell-free systems (36-fold), but not cyclic nucleotide-dependent protein kinases (approximately 2-fold for both PKA and PKG) (Kase *et al,* 1986; Tamaoki *et al,* 1986). In addition, LHRH priming was inhibited by Ro 31-8220 (which has been reported by Davis and colleagues (1989) to be 2 - 3 orders of magnitude more effective at inhibiting PKC than PKA or Ca^{2+}/calmodulin-dependent protein kinase) and this inhibition of priming occurred at similar concentrations (IC_{50} = 19.0 ± 6.8 μM) to the inhibition of 100 nM PDBu-induced LH release (IC_{50} = 15.6 ± 4.5 μM). Inhibitors that show selectivity for other protein kinases over PKC, such as tyrosine kinase inhibitors, calmodulin antagonists and inhibitors of the myosin light chain kinase family are unable to prevent the LHRH priming response when used at concentrations greater than their respective IC_{50} values in whole cells (Johnson *et al,* 1992a). In fact, in these experiments, LHRH-induced secretion was enhanced in the presence of the calmodulin antagonists, and others have shown that these drugs can facilitate PMA-induced LH release (Iwashita *et al,* 1984). Although certain calmodulin inhibitors can inhibit LHRH responses in dispersed cells (Conn *et al,* 1981b), these agents can also alter surface membrane potential (McLaughlin and Whitaker, 1988) and bind to proteins other than calmodulin (Moor and Dedman, 1982), and these non-specific actions may contribute to the effects of these drugs on LHRH responses (Van der Merwe *et al,* 1990a). Circumstantial evidence also suggests that the cyclic nucleotide-dependent kinases are not involved in LHRH responses. Both LHRH-induced LH release and priming can be uncoupled from changes in gonadotroph cAMP levels and can only be mimicked by very high, possibly toxic levels of dibutyryl cAMP (Naor *et al,* 1975b, 1978; Conn *et al,* 1979; Pickering and Fink, 1979), arguing against PKA involvement in LHRH responses. Cyclic GMP analogues do not induce LH release from anterior pituitary (Naor and Catt, 1980; Liu and Jackson, 1981), arguing against a role for PKG in LHRH responses. Finally, PKC activator-induced LH release was seen to develop with each

consecutive hourly incubation (Figures 3.5 and 3.7), thus activation of PKC with phorbol esters can mimic the temporal profile of LHRH priming response.

Protein kinase C translocation and activation can occur within minutes of an LHRH stimulus (Hirota *et al,* 1985). However, several pieces of evidence presented in this chapter suggest that PKC activation is not essential for initial responses to LHRH, but is required for the induction of LHRH priming in pro-oestrous rat hemipituitaries. Firstly, PKC inhibitors had no effect on initial LHRH-induced gonadotrophin release, but inhibited primed release to a level equivalent to the initial response, but never to basal levels (Figures 3.2 and 3.3). In a similar manner, staurosporine and another putative PKC inhibitor, retinal, can block late response to LHRH in dispersed anterior pituitary cells without having any effect on acute LHRH-induced LH release (Chang *et al,* 1987b; Stojilkovic *et al,* 1991). In pro-oestrous rat hemipituitaries, staurosporine exerted its inhibitory effects on primed gonadotrophin release if present during the hour in which LHRH priming had taken place (i.e. the 1st h, Figures 3.1, 3.2, 3.3, 3.4a), indicating that PKC activation is most likely to be required for the induction of LHRH priming. Furthermore, the response of previously-primed tissue to LHRH was unaltered by staurosporine (Figure 3.4b), further suggesting that PKC activation is not required for the expression, but instead, is needed for the induction of priming. Staurosporine did not inhibit LH release induced by ionomycin (Table 3.1), indicating that this inhibitor did not have a delayed, non-specific action on the secretory apparatus of the gonadotroph. However, staurosporine could prevent the LHRH-induced change in pituitary responsiveness, even when LH release from previously-primed cells was elicited by ionomycin rather than a second LHRH challenge, further indicating that PKC activation is specifically required for the induction of priming. These results are consistent with observations made by other workers who have found acute LHRH-induced LH release to be independent of PKC activation (McArdle *et al,* 1987; Stojilkovic *et al,* 1991). One isolated report has suggested that staurosporine can enhance early responses to LHRH

(Van der Merwe *et al,* 1990b). However, this result may be explained by the way the authors expressed LH release, i.e. as a percentage of the total LH present after an initial stimulus. Luteinizing hormone-releasing hormone can induce a PKC-dependent synthesis of additional LH, elevating total pituitary gonadotrophin content (Andrews *et al,* 1988; Stojilkovic *et al,* 1988b). Staurosporine may, therefore, prevent LHRH-induced gonadotrophin synthesis, but not initial secretory responses to LHRH and as a consequence, the response to LHRH, may appear to be enhanced in the presence of this inhibitor.

Activators of PKC can mimic the late component of LHRH-induced LH release in pro-oestrous pituitary pieces (Figure 3.4; Turgeon and Waring, 1986) consistent with PKC involvement in later responses to LHRH. Furthermore, pre-treatment of anterior pituitary pieces with PKC activators can enhance subsequent responses to LHRH (Johnson *et al,* 1988), suggesting that activation of PKC can increase pro-oestrous rat gonadotroph responsiveness, perhaps by inducing the priming mechanism independently of LHRH receptor activation. The delay between PKC activation and expression of the primed response indicates that there may be several steps in the process by which PKC activation can result in LHRH priming.

The data presented in Figure 3.2 show that LHRH priming is attenuated by H7, but with lower potency ($IC_{50} = 71.6 \pm 13.3$ μM) than has been reported previously for the actions of this drug on PKC-mediated cellular responses (Hidaka *et al,* 1984). The simplest interpretation of these results would be that LHRH priming involves a PKC-like kinase which is relatively H7-resistant. Additional evidence to support this hypothesis was obtained by examining the effect of H7 on PKC activator-induced LH and GH release. In these experiments, mezerein- and PDBu-induced LH release consisted of both H7-sensitive and -insensitive components (Figures 3.9 and 3.10) and DOG-induced LH release consisted of the H7-resistant component only (Table 3.3). Mezerein and PDBu may, therefore, activate H7-sensitive PKCs, in addition to the H7-resistant PKC(s) that is involved in priming, to induce LH release.

Although LHRH priming involves the H7-resistant PKC(s) only, the H7-sensitive PKC forms may indirectly contribute to LH release by modulating other factors which are involved in the LH release process. For example, PKC activators can promote Ca^{2+} entry through voltage-sensitive Ca^{2+} channels of gonadotrophs and thereby affect hormone release in general (Stojilkovic *et al,* 1988a, 1991; Izumi *et al,* 1990). Protein kinase C activators can also induce synthesis of new gonadotrophin (Andrews *et al,* 1988; Stojilkovic *et al,* 1988b).

Phorbol 12,13-dibutyrate-induced GH release from pro-oestrous rat anterior pituitary tissue was also resistant to block by H7 at concentrations up to 30 μM. Although H7 inhibits other serine-threonine kinases in addition to PKC (IC_{50} values: PKG, 6 μM; PKA, 3 μM; myosin light chain kinase, 97 μM; and PKC, 6 μM) (Hidaka *et al,* 1984) there is no explicit evidence to suggest that DAGs, phorbol esters or phorbol-related compounds, such as mezerein, can directly activate protein kinases other than PKC. As a consequence, the H7-resistant kinase observed in these experiments is most probably PKC or a PKC-like kinase. Additional evidence that pro-oestrous rat anterior pituitary cells express H7-resistant PKCs has come from experiments where partially purified cytosolic PKC activity was measured in a mixed micelle assay using histone as a substrate (Table 3.4). Phosphatidylserine-dependent, PDBu-stimulated kinase activity consisted of a Ca^{2+}-independent component which was relatively H7-resistant, but readily blocked by staurosporine. In contrast, Ca^{2+}-dependent PDBu-induced PKC activity displayed the expected sensitivities to inhibition by H7 and staurosporine. Several other reports have also described phorbol ester responses which display high resistance to inhibition by H7, but not staurosporine (Watson *et al,* 1988; Johnson and Mitchell, 1989; Johnson *et al,* 1989; Nakadate *et al,* 1989; Fink *et al,* 1990; MacEwan and Mitchell, 1991) and since H7 and its closely related congener, HA 1004, show relative selectivity between different classes of kinases (Hidaka and Hagiwara, 1987), it would be reasonable to postulate that H7 might also distinguish between different subtypes of PKC.

Although H7 and staurosporine are structurally different, both compounds are thought to inhibit PKC activity by binding at or near to the ATP binding site, hence the contrast between the effects of H7 and staurosporine on LHRH priming and on PKC activator-induced GH and LH release is, at first, unexpected. However, several lines of evidence suggest that the sites of action of these two inhibitors are not identical. Firstly, H7, but not staurosporine, has been reported to act in a kinetically competitive manner with ATP (Hidaka *et al,* 1984; Tamaoki *et al,* 1986; Rüegg and Burgess, 1989) although other evidence does show apparent competitive mechanisms for staurosporine and K252a (Kase *et al,* 1986; Davis *et al,* 1989). Secondly, the binding of [^{3}H]-DMS is displaced by H7 with extremely low potency (IC_{50} > 500 μM) (see Chapter 6). Furthermore, the α and β PKC isoforms have both been reported to have a second consensus recognition motif for ATP (Huang, 1989), indicating that the interactions between H7 and these isoforms may be complex.

Several earlier reports have shown that different classes of PKC activator can induce LH and GH release from anterior pituitary tissue *in vitro* and from anterior pituitary cells in culture (Smith and Vale, 1980; Conn *et al,* 1985; Nigro-Villar and Lapetina, 1985; Turgeon and Waring, 1986; Boyd and Wallis, 1989). However, the data presented here indicates that the kinases which induce LH and GH release have distinct pharmacological profiles with respect to different PKC activators. Growth hormone release was induced by mezerein and PDBu with equal potency suggesting that the PKC(s) involved are similarly activated by both of these compounds. In contrast, LH release was induced more effectively by mezerein than PDBu, suggesting that the PKCs involved in this process are more readily activated by mezerein. Differences in the tumour promoting effects of phorbol esters, such as PMA, and mezerein have been reported previously (Slaga *et al,* 1980) and may reflect the ability of these compounds to preferentially activate certain PKC forms.

1,2-Dioctanoyl-*sn*-glycerol was unable to induce any significant release of GH and although DOG could enhance LH release, the secretory response to DOG was

much smaller than that measured using either PDBu or mezerein. In certain other models of PKC function in pituitary cells, for example, the facilitation of depolarisation-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms, a DOG concentration of 100 μM elicited effects of equivalent magnitude to 300 nM PDBu or mezerein. The low potency of DOG on LH release, and in the $^{45}Ca^{2+}$ model suggests that DOG may selectively activate certain PKC forms (MacEwan and Mitchell, 1991). Indeed, in other physiological models of PKC action, DOG has been shown to mimic some, but not all, actions of phorbol esters (Lacerda *et al,* 1988, see also Chapters 4 and 5). Certainly, it appears that DOG can activate an H7-resistant PKC(s) that is involved in PKC activator-induced LH release. Reduced potency of DOG as an activator of α-PKC isoform has been reported (Sekiguchi *et al,* 1988; MacEwan *et al,* 1992a); an effect which is most marked under conditions of basal cytosolic Ca^{2+} (≤ 100 μM). However, DOG, unlike PDBu and mezerein, is susceptible to intracellular degradation by DAG lipases (Kaibuchi *et al,* 1983), which may also partly contribute the low efficacy of DOG on LH release, especially in the relatively long incubation periods used here.

Different PKC isoforms have been implicated as having distinct roles in certain cellular responses. For example, during thyrotrophin-releasing hormone (TRH) stimulation of the anterior pituitary derived GH_4 C_1 cell line (Kiley *et al,* 1991, 1992) the α, β, δ and ε PKC isoforms associate with the cytoskeleton causing cytoskeletal changes. However, selective down-regulation of the ε isoform did not alter the regulatory effects of TRH on actin filament organisation, suggesting that an isoform, other than ε PKC, is involved in this process. The relationship of the PKC(s) forms involved in LHRH priming, and LH and GH release in pro-oestrous anterior pituitary tissue to the characterized PKC isoforms is not entirely clear from the results presented here. Of the known PKC types, the α, β_{II}, ε, but not γ nor β_I isoforms are expressed in anterior pituitary (Naor *et al,* 1988; Naor, 1990). Northern blot analysis has indicated that the mRNA for the ε and also the ζ but perhaps not the δ isoform are

present in significant levels (Lutz, Ison and Mitchell, personal communication) but it is unclear whether or not anterior pituitary cells express the η, λ or θ PKC isoforms. When added to permeabilized pituitary cells, both α and β isoforms can stimulate LH release (Naor *et al,* 1989). Certainly, the H7-sensitive PKC(s) involved in LH release in pro-oestrous rat tissue may be one or more of the Ca^{2+}-dependent types, such as the α and β isoforms. However, Ca^{2+}-independent, H7-resistant PKCs are also involved in LH release, GH release, and LHRH priming and therefore, may include one or more of the (δ), ε, ζ, η, λ or θ isoforms. Data obtained using expressed and purified enzymes have shown that the ε and δ-isoforms of PKC display the expected degree of sensitivity to H7 (Schaap and Parker, 1990; Uchida *et al,* 1991), indicating that neither ε nor δ PKC may be the H7-resistant PKC(s) shown here. In addition, the ζ and λ PKC isoforms are reported to be phorbol ester-insensitive (Liyanage *et al,* 1992; Y. Nishizuka, personal communication) suggesting that neither of these isoforms represents the PDBu-induced, H7-resistant kinase activity detected in anterior pituitary cytosol (Table 3.4). Since histone has been reported to be a poor substrate for the characterised Ca^{2+}-independent isoforms (Ohno *et al,* 1988; Huang, 1989; Schaap *et al,* 1989; Schaap and Parker, 1990; Olivier and Parker, 1991), the H7-resistant, Ca^{2+}-independent, histone kinase activity in anterior pituitary cytosol seems unlikely to represent any of the known Ca^{2+}-independent isoforms. There are a number of complicating factors, however, since there is increasing evidence that the degree of Ca^{2+}-dependency of the α, β and γ isoforms, may alter in the presence of certain fatty acids (Murakami and Routtenberg, 1985; Naor *et al,* 1988; Shinomura *et al,* 1991) and that the Ca^{2+} dependency of the β isoform can be altered by its phosphorylation state (Pelech *et al,* 1991). Using purified/expressed enzyme preparations, the known PKC isoforms also differ in their PKC activator pharmacology (Evans *et al,* 1991; Ryves *et al,* 1991), but it is difficult to relate these observations made under fixed assay conditions with differences seen in whole cells (as has been shown in this study). For example, the choice of substrate used in an

assay can determine the Ca^{2+} dependency of the α, β and γ isoforms and can alter the potency of DAGs and phorbol esters as PKC activators (Wolf *et al,* 1984; Bazzi and Nelsestuen, 1987). In addition, DAGs stimulate various PKC isozymes with different potency in assays containing different phospholipids (Huang *et al,* 1988). Therefore the activator pharmacology of cellular PKCs will probably vary according to the phospholipid environment and substrate.

In comparison to LHRH receptor signalling, the intracellular changes which occur upon GH-releasing hormone (GHRH) receptor activation are much less well understood. Growth hormone-releasing hormone does not stimulate inositol phospholipid turnover (Collado Escobar *et al,* 1986; French *et al,* 1990) and PKC activation is not required for the actions of GHRH (Ohmura and Friesen, 1985; French *et al,* 1989). Growth hormone-RH stimulation of somatotrophs is accompanied, instead, by stimulation of adenylate cyclase activity (Labrie *et al,* 1983) and a subsequent increase in cAMP levels (Bilezikjian and Vale, 1983). Although PKC activation may not be essential for the actions of GHRH, PKC may facilitate cAMP production and GH release in response to GHRH (Summers *et al,* 1985; Cheng *et al,* 1991). Thus, the actions of PKC in GHRH receptor signalling may bear some analogy to the facilitatory effects of PKC on LHRH-induced gonadotrophin release reported here.

In summary, it has been shown here that several pharmacologically distinct PKC forms exist in anterior pituitary tissue and these forms differentially control LH release, GH release and LHRH priming in pro-oestrous rat tissue pieces *in vitro*. Although these experiments provide preliminary evidence regarding the relationship of these pharmacologically distinct forms to the known PKC isoforms, further studies are required to define their precise identity. Since the known PKC isoforms have distinct tissue distributions, it may be possible to correlate the distribution of the H7-resistant kinase(s) described here with the tissue distribution of the known PKC forms. For example, the H7-sensitivity of PKC activity purified from

different tissue sources may be determined and compared using the kinase assay method described here. However, since the H7-resistant PKC may represent a modified version of a known PKC isoform, an alternative approach may be required to identify this kinase. Using isoform-specific antibodies, one could immunodeplete certain PKC isoforms from cell free preparations of anterior pituitary PKC in an attempt to identify an H7-resistant activity. One could also attempt to inhibit LHRH priming using PKC isoform-selective antibodies, although this approach requires dispersed, permeabilised cells which clearly do not function in an identical manner to whole tissue (see section 1.4.3). Antisense oligonucleotides to specific PKC isoforms may also be used to inhibit LHRH priming although this approach is limited since it is dependent upon the gene encoding the kinase being active. Since the structure of the pseudosubstrate region of different PKC isoforms may show heterogeneity, it is possible that isoform-selective inhibitors may be developed which target this region. Such compounds may, potentially, help to clarify the identity of the PKC involved in LHRH priming. However, it is possible that some of the pharmacological differences that we are observing here may represent the involvement of new, as of yet unidentified PKC isoforms or PKC-like kinase.

The different temporal pattern of PKC activator-induced LH and GH release indicates that these distinct PKCs may also phosphorylate different cellular targets. Further experiments to determine the cellular targets of the PKC forms involved in LHRH priming and PKC activator-induced LH and GH release will be of interest (see Chapter 4).

FIGURE 3.1

The effect of staurosporine on LHRH-induced (a) LH and (b) FSH release from pro-oestrous rat hemipituitaries

Control treatments (open bars) were incubated for consecutive hours in the presence of medium only (basal h) followed by consecutive periods (1st h, 2nd h) in the presence of LHRH (1 nM). Including staurosporine (300 nM) throughout each hour (hatched bars) had no significant effect on basal h or 1st h LHRH-induced LH or FSH release. The release of both LH and FSH measured during the 2nd h was significantly inhibited in the presence of staurosporine ($*p < 0.05$). Data are mean ± SEM for 4 - 6 determinations.

Figure 3.1

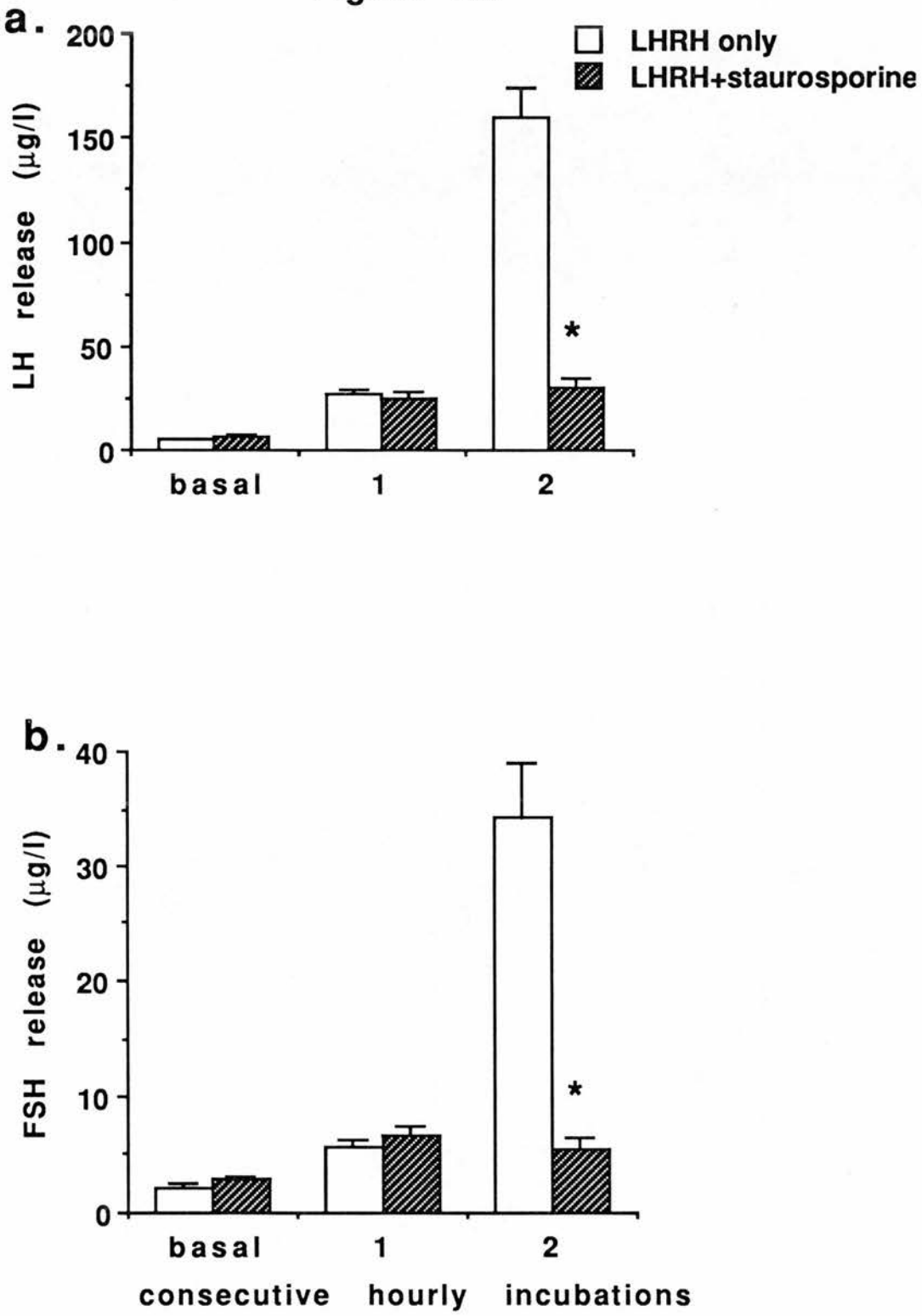

a.
LHRH only
LHRH+staurosporine
LH release (µg/l)
200
150
100
50
0
*
basal
1
2
b.
FSH release (µg/l)
40
30
20
10
0
*
basal
1
2
consecutive
hourly
incubations

FIGURE 3.2

Concentration-effect curves for the action of the PKC inhibitors, (a) staurosporine, (b) K252a, (c) Ro 31-8220 and (d) H7 on LHRH-induced LH release from pro-oestrous rat hemipituitaries

After a basal hour incubation with PKC inhibitor only, tissue was incubated for consecutive hours (1st h, 2nd h) in the presence of LHRH (1 nM) and PKC inhibitor. First h LHRH-induced LH release was unaffected by either staurosporine (10 - 1000 nM), K252a (0.03 - 10 μM), Ro 31-8220 (0.1 - 50 μM) or H7 (10 - 300 μM). However, 2nd h LHRH-induced LH release was significantly inhibited by all four compounds when compared to 2nd h LHRH-induced LH release in the absence of PKC inhibitor (*$p < 0.05$). The IC_{50} values for inhibition of the 2nd h response were 26.3 ± 7.0 nM for staurosporine, 2.9 ± 0.4 μM for K252a, 19.0 ± 6.8 μM for Ro 31-8220 and 71.6 ± 13.3 μM for H7. Each point represents the mean ± SEM for 4 - 6 determinations.

Figure 3.2

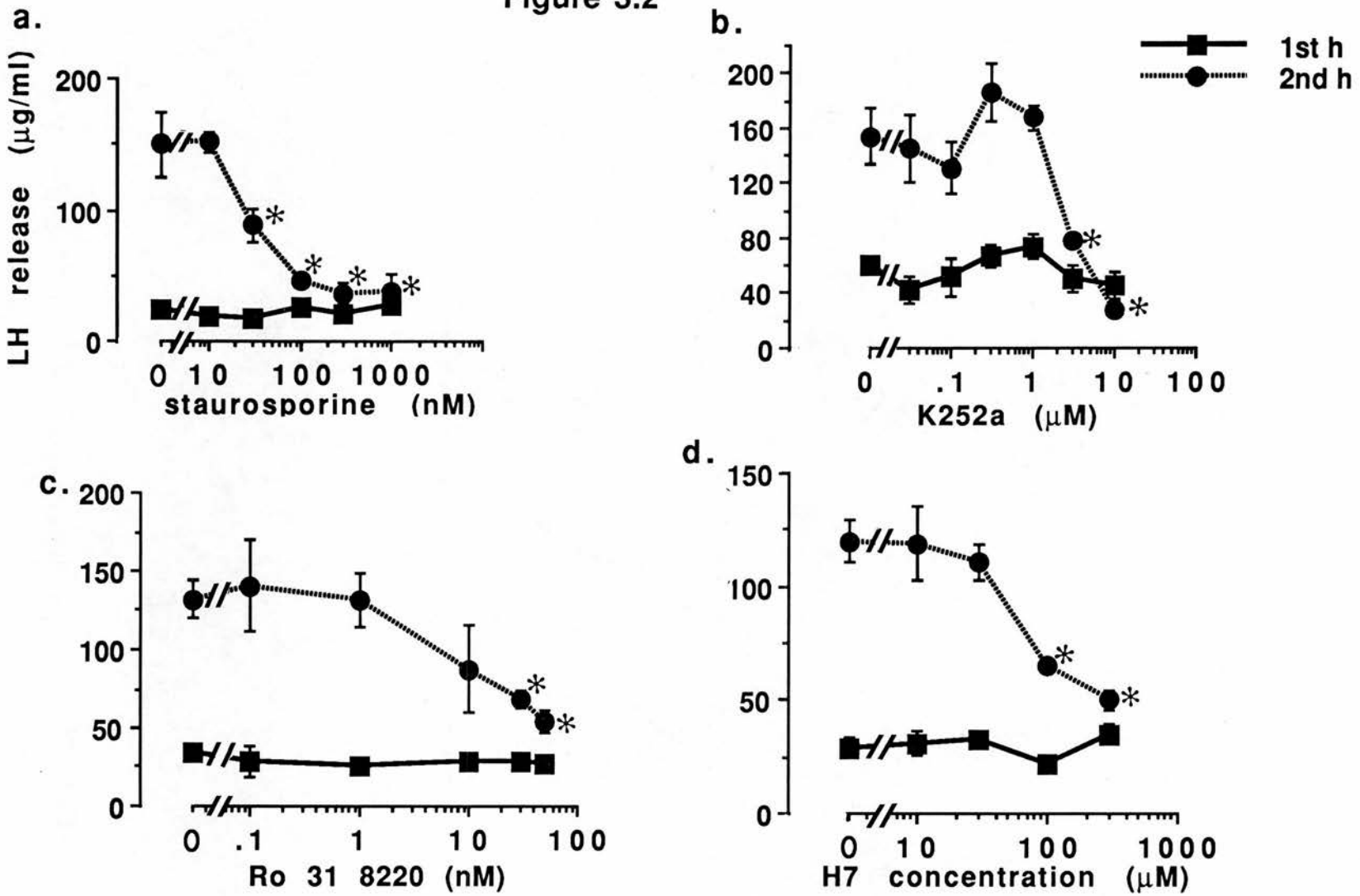

a.
LH release (µg/ml)
200
100
0
0 10 100 1000
staurosporine (nM)
b.
1st h
2nd h
200
160
120
80
40
0
0 .1 1 10 100
K252a (µM)
c.
200
150
100
50
0
0 .1 1 10 100
Ro 31 8220 (nM)
d.
150
100
50
0
0 10 100 1000
H7 concentration (µM)

FIGURE 3.3

Concentration-effect curves for the action of the PKC inhibitors (a) staurosporine, (b) K252a and (c) H7 on LHRH-induced FSH release from pro-oestrous rat hemipituitaries

After a basal hour incubation with PKC inhibitor only, tissue was incubated for consecutive hours (1st h, 2nd h) in the presence of LHRH (1 nM) and PKC inhibitor. first h LHRH-induced FSH release was unaffected by staurosporine (10 - 300 μM), K252a (0.03 - 10 μM) or H7 (10 - 300 μM). However, 2nd h LHRH-induced FSH release was significantly inhibited by all three compounds when compared to 2nd h LHRH-induced FSH release in the absence of PKC inhibitor (*$p < 0.05$). The IC_{50} values for inhibition of the 2nd h response were 37.0 ± 7.1 nM for staurosporine, 2.3 ± 0.5 μM for K252a and 88.1 ± 20.4 μM for H7. Each point represents the mean SEM for 4 - 6 determinations.

Figure 3.3

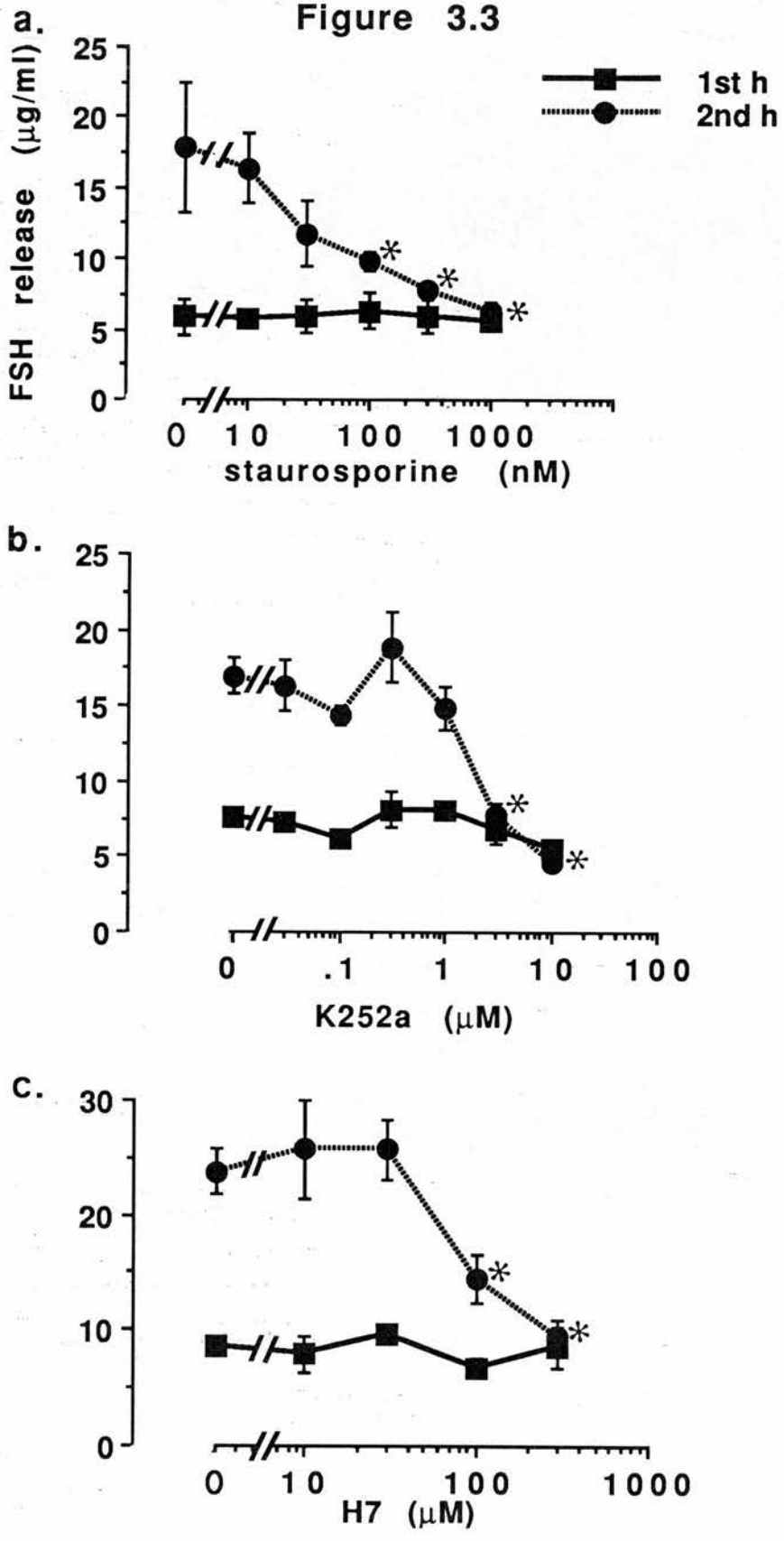

a.
FSH release (μg/ml)
25
20
15
10
5
0
1st h
2nd h
*
*
*
0 10 100 1000
staurosporine (nM)
b.
25
20
15
10
5
0
*
*
0 .1 1 10 100
K252a (μM)
c.
30
20
10
0
*
*
0 10 100 1000
H7 (μM)

FIGURE 3.4

The effect of staurosporine on the development of LHRH priming in pro-oestrous rat hemipituitaries

In part **a**, tissue was incubated with staurosporine (300 nM) for an initial basal h and in the 1st h together with LHRH (1 nM) (hatched bars). Tissue was then extensively washed before receiving a final hour incubation (2nd h) in the presence of LHRH only (open bars). Although staurosporine was not present during the 2nd h, primed release was significantly inhibited (*$p < 0.05$). Control samples received LHRH only in the 1st h and 2nd h. Part **b** shows the effect of staurosporine on LHRH-induced LH release from pre-primed pro-oestrous rat hemipituitaries. Following a basal h incubation in medium only, tissue was primed by incubating with LHRH (1 nM) for 1h. Tissue was then exposed to two further consecutive hourly incubations (2nd h, 3rd h) either with LHRH only (open bars) or LHRH and staurosporine (hatched bars). Staurosporine (300 nM) had no significant inhibitory effect on LHRH-induced LH release from previously primed tissue. Data are mean ± SEM for 8 determinations.

Figure 3.4

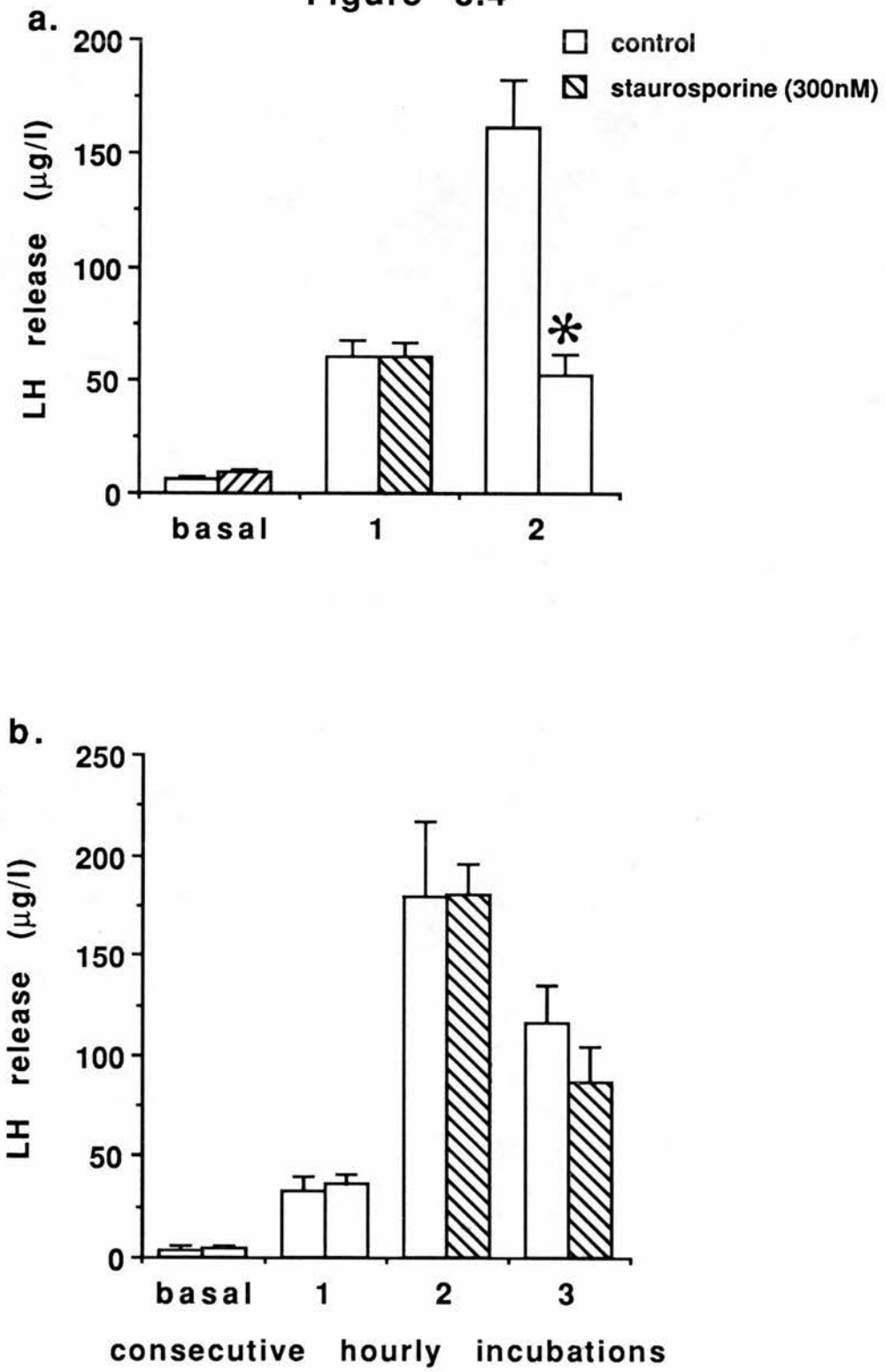

a.
control
staurosporine (300nM)
LH release (µg/l)
200
150
100
50
0
*
basal
1
2
b.
LH release (µg/l)
250
200
150
100
50
0
basal
1
2
3
consecutive hourly incubations

FIGURE 3.5

Temporal profile of the effect of phorbol 12,13-dibutyrate (PDBu) on LH and GH release from pro-oestrous rat anterior pituitary tissue

Hemipituitary pieces were incubated for an initial hour in the presence of medium only (basal h) followed by consecutive hours with 100 nM PDBu (1st h, 2nd h, 3rd h). The release of GH (hatched bars) was maximal in the 1st h of incubation with PDBu whereas release of LH (open bars) was only significantly different (*$p < 0.05$) from basal in the 2nd h with PDBu and continued to rise in the 3rd h (**$p < 0.01$). Values are mean ± SEM, n = 5 - 11.

Figure 3.5

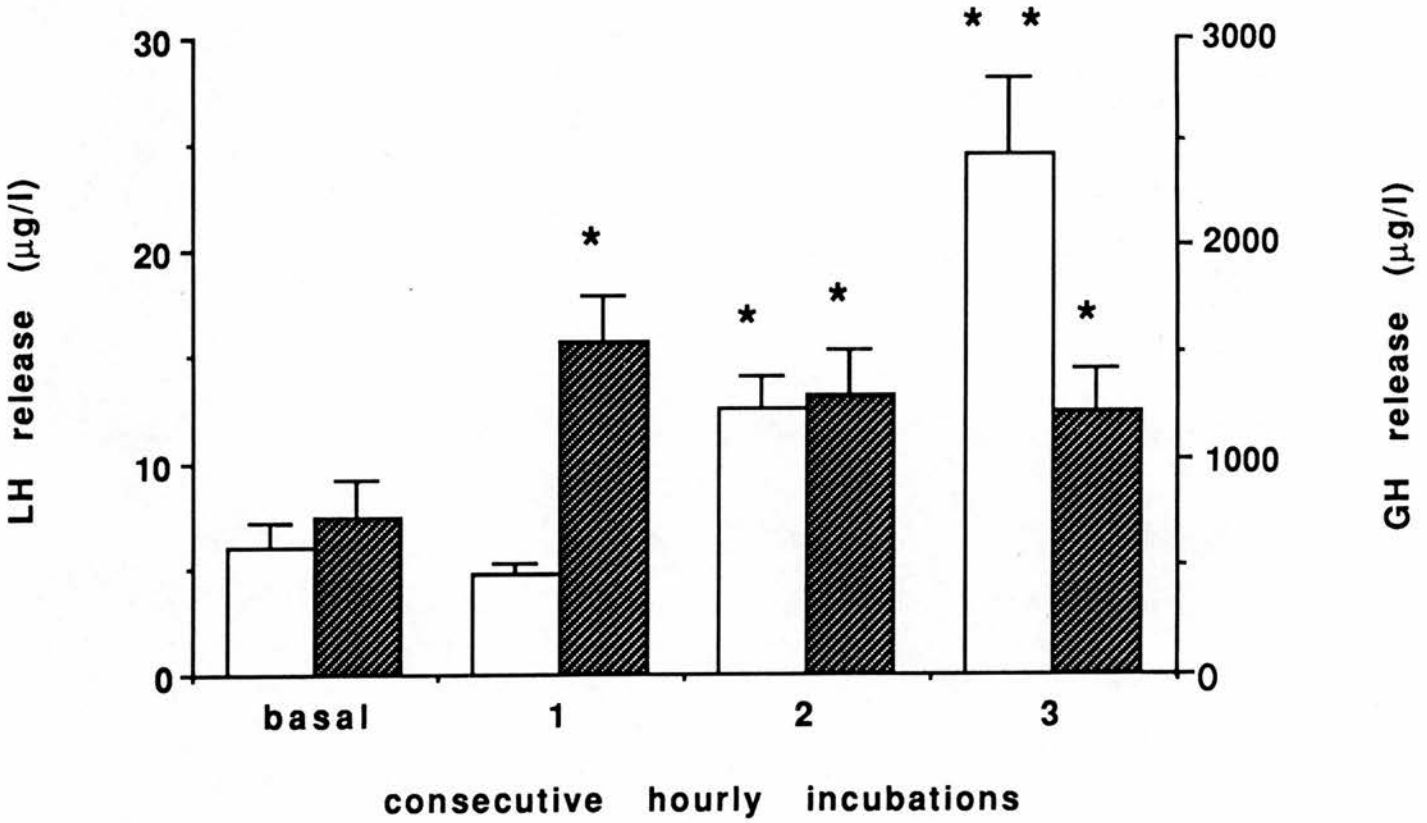

LH release (µg/l)
GH release (µg/l)
30
20
10
0
3000
2000
1000
0
basal
1
2
3
consecutive hourly incubations
*
*
*
*
*
*

FIGURE 3.6

Concentration response curves for the effect of phorbol 12,13-dibutyrate (■) and mezerein (●) on (a) LH and (b) GH release from pro-oestrous rat hemipituitaries

Tissue was incubated for a basal h in medium only followed by consecutive hours in the presence of either PDBu (0.03 - 1 μM) or mezerein (0.03 - 1 μM). These graphs show LH release measured in the 3rd h of incubation and GH release during the 1st h of incubation. Data are expressed as the fold of release that occurred in the initial basal h. Each point on the graphs represents n = 4 - 11.

Figure 3.6

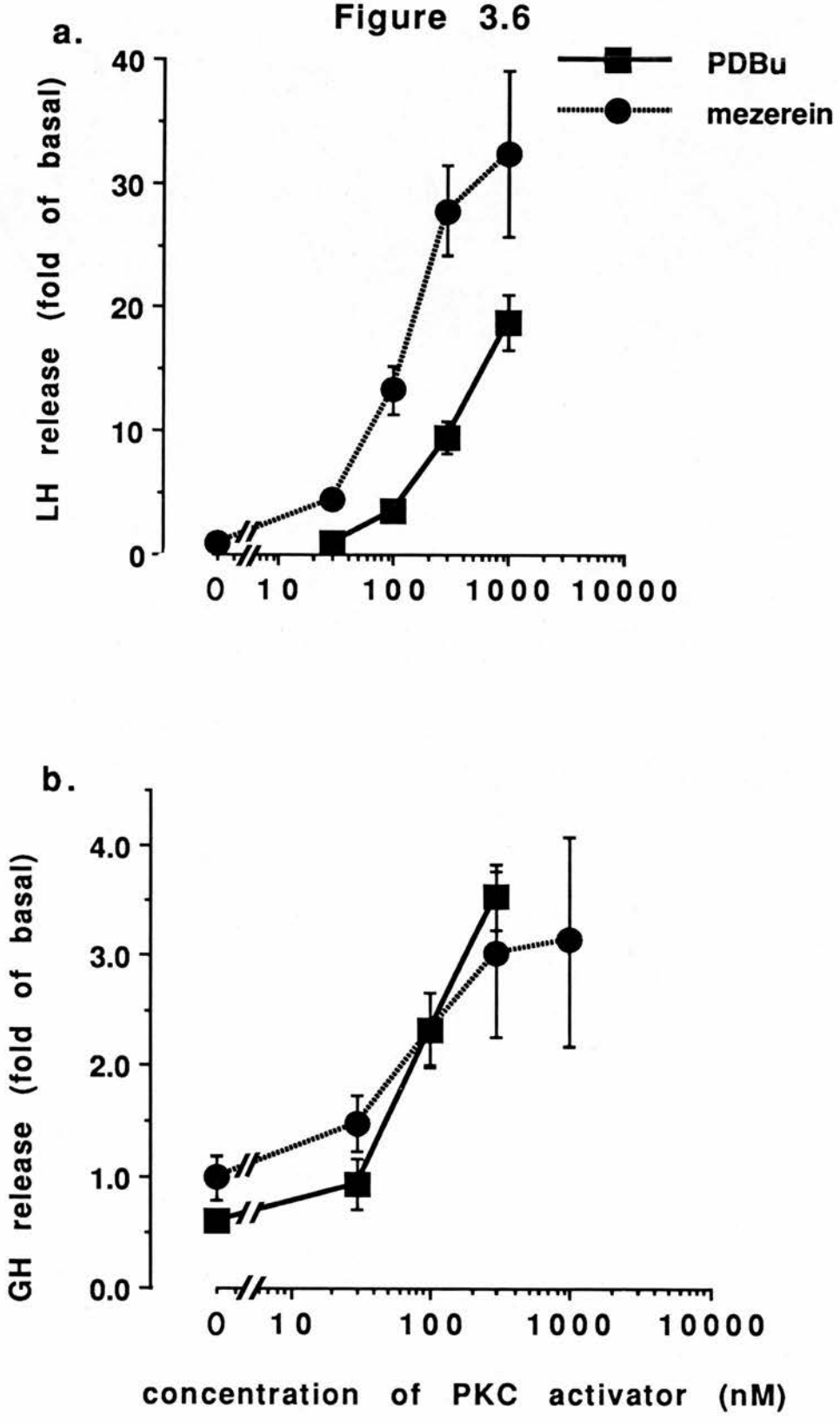

a.
LH release (fold of basal)
40
30
20
10
0
0 10 100 1000 10000
PDBu
mezerein
b.
GH release (fold of basal)
4.0
3.0
2.0
1.0
0.0
0 10 100 1000 10000
concentration of PKC activator (nM)

FIGURE 3.7

The temporal pattern of 1,2-dioctanoyl-*sn*-glycerol (DOG)-induced (a) LH and (b) GH release from pro-oestrous rat anterior pituitary pieces

Tissue was incubated for 4 consecutive hours in the presence of medium only (baseline, open bars) followed by consecutive hours in the presence of 200 μM DOG (hatched bars). Luteinizing hormone, but not GH, release was significantly greater than baseline levels throughout the 2nd and 3rd h incubation with DOG (*$p < 0.05$). However, GH release was not significantly different from baseline levels during any hour of incubation with DOG. Values are means ± SEM for 4 - 6 determinations.

Figure 3.7

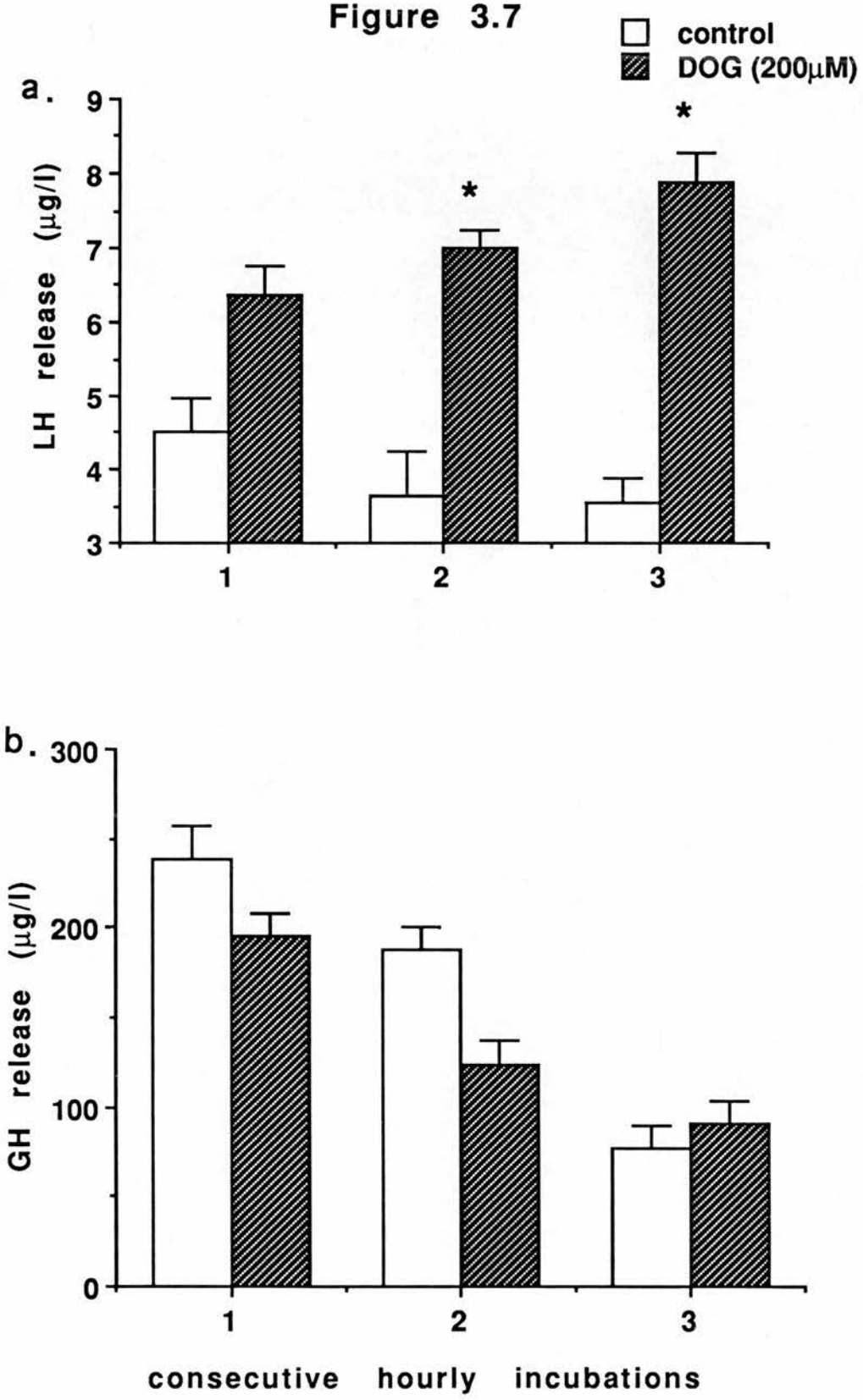

control
DOG (200μM)
a.
LH release (μg/l)
9
8
7
6
5
4
3
*
*
1
2
3
b.
GH release (μg/l)
300
200
100
0
1
2
3
consecutive hourly incubations

FIGURE 3.8

Concentration-response curves for the effects of (a) staurosporine on phorbol 12,13-dibutyrate (PDBu)-induced LH (●) and GH release (■) from pro-oestrous rat anterior pituitary tissue

Tissue was incubated for a basal hour in medium only or together with staurosporine (10 - 300 nM), followed by consecutive hourly incubations (1st h, 2nd h, 3rd h), in addition, with PDBu (100 nM). These graphs show LH release measured in the 3rd h of incubation and GH release during the 1st h of incubation. Staurosporine blocked 100 nM PDBu-induced LH and GH release with similar effectiveness. Data are shown as mean ± SEM, n = 4 - 6.

Figure 3.8

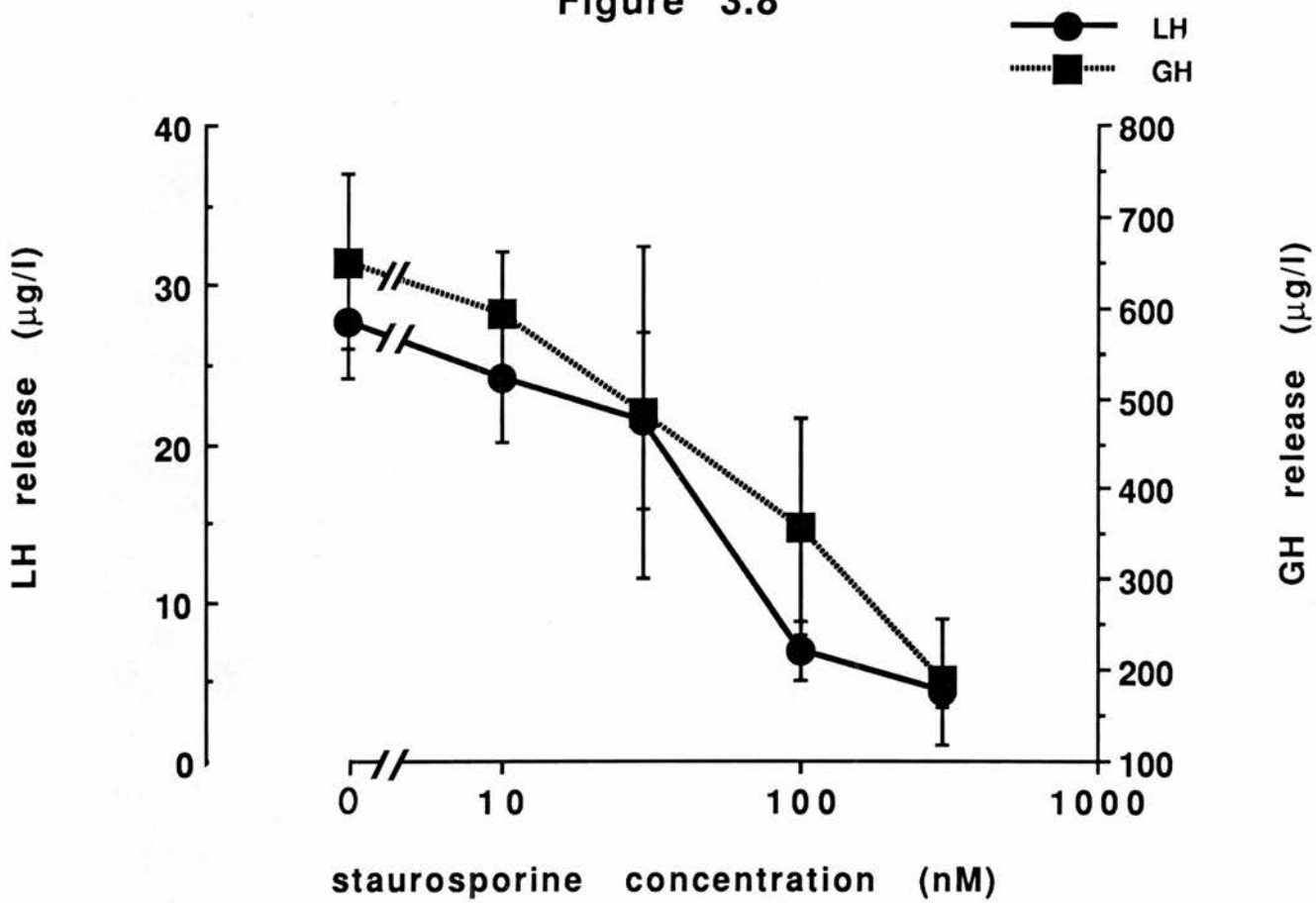

LH
GH
LH release (µg/l)
GH release (µg/l)
40
30
20
10
0
800
700
600
500
400
300
200
100
0
10
100
1000
staurosporine concentration (nM)

FIGURE 3.9

Concentration-response curves for the effect of (a) H7 on phorbol 12,13-dibutyrate (PDBu)-induced LH and GH release and (b) H7 on mezerein-induced LH and GH release from pro-oestrous rat anterior pituitary tissue

Tissue was incubated for consecutive hours (basal h, 1st h, 2nd h, 3rd h) in the presence of various concentrations of H7. In addition, either 100 nM PDBu (a) or 300 nM mezerein (b) was also present throughout the 1st h, 2nd h and 3rd h incubations. The data show GH (■) release measured during the 1st h and LH release (●) measured in the 3rd h of incubation with PKC activators. The statistical significant inhibition of hormone release by H7 is shown by *($p < 0.05$). The corresponding basal release of LH during the 3rd h of drug-free controls was 2.5 ± 0.3 μg/l (a) and 3.9 ± 0.4 μg/l (b). H7 alone had no effect on basal release of LH or GH at any of the concentrations tested. Each point on the graphs represents the mean ± SEM for 4 - 6 determinations.

Figure 3.9

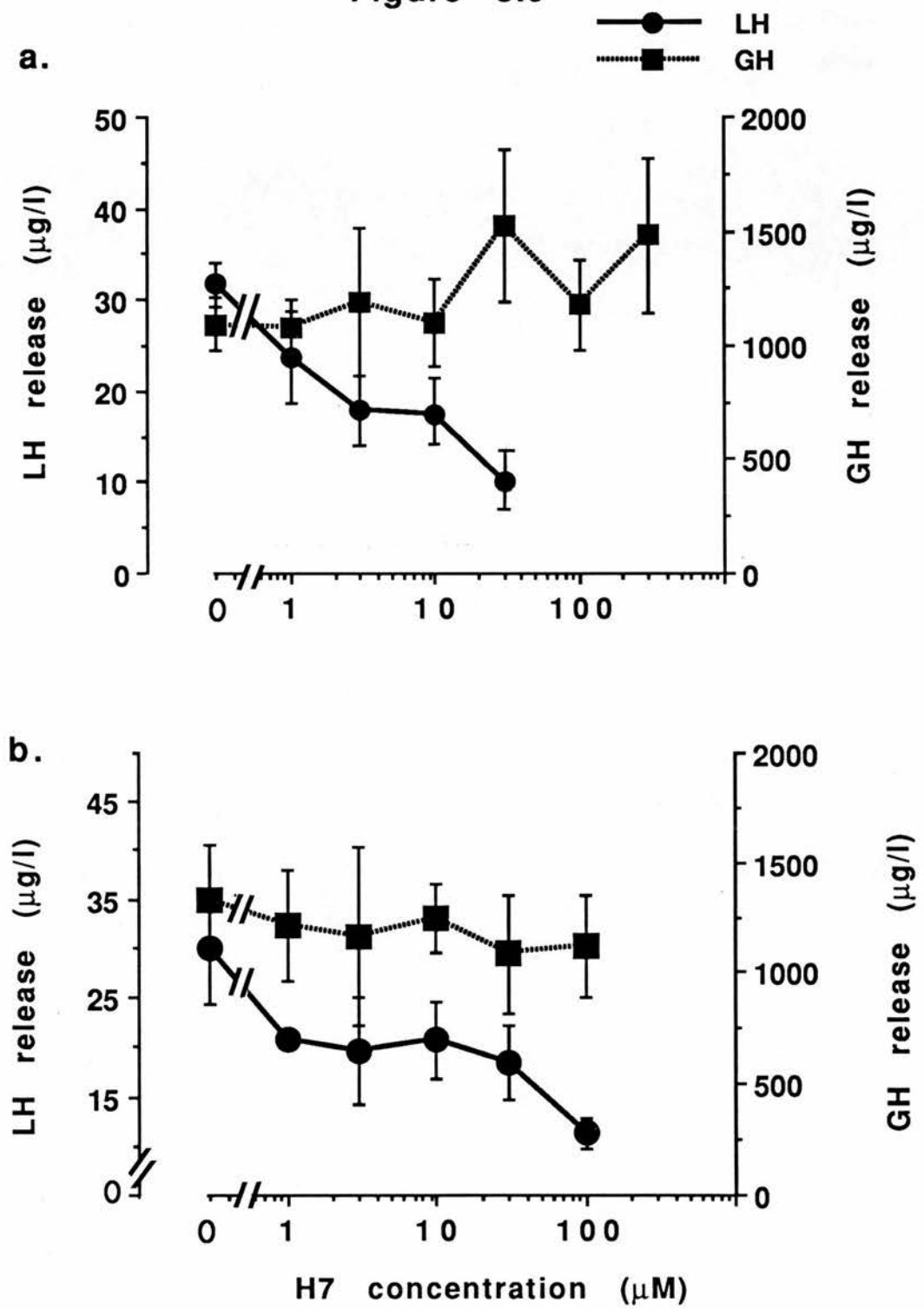

a.
LH
GH
LH release (μg/l)
GH release (μg/l)
50
40
30
20
10
0
2000
1500
1000
500
0
0
1
10
100
b.
45
35
25
15
0
2000
1500
1000
500
0
0
1
10
100
H7 concentration (μM)

FIGURE 3.10

The effect of H7 on LH release induced from pro-oestrous rat anterior pituitary tissue by increasing concentrations of phorbol 12,13-dibutyrate (PDBu)

Tissue was incubated for consecutive hours with various concentrations of PDBu (3 - 1000 nM) either in the presence or absence of H7 (30 μM). The data shows the % of the LH response, measured during the 3rd h of PDBu incubation, that was inhibited by H7. Each point on the graph represents the mean ± SEM for 4 - 6 determinations.

Figure 3.10

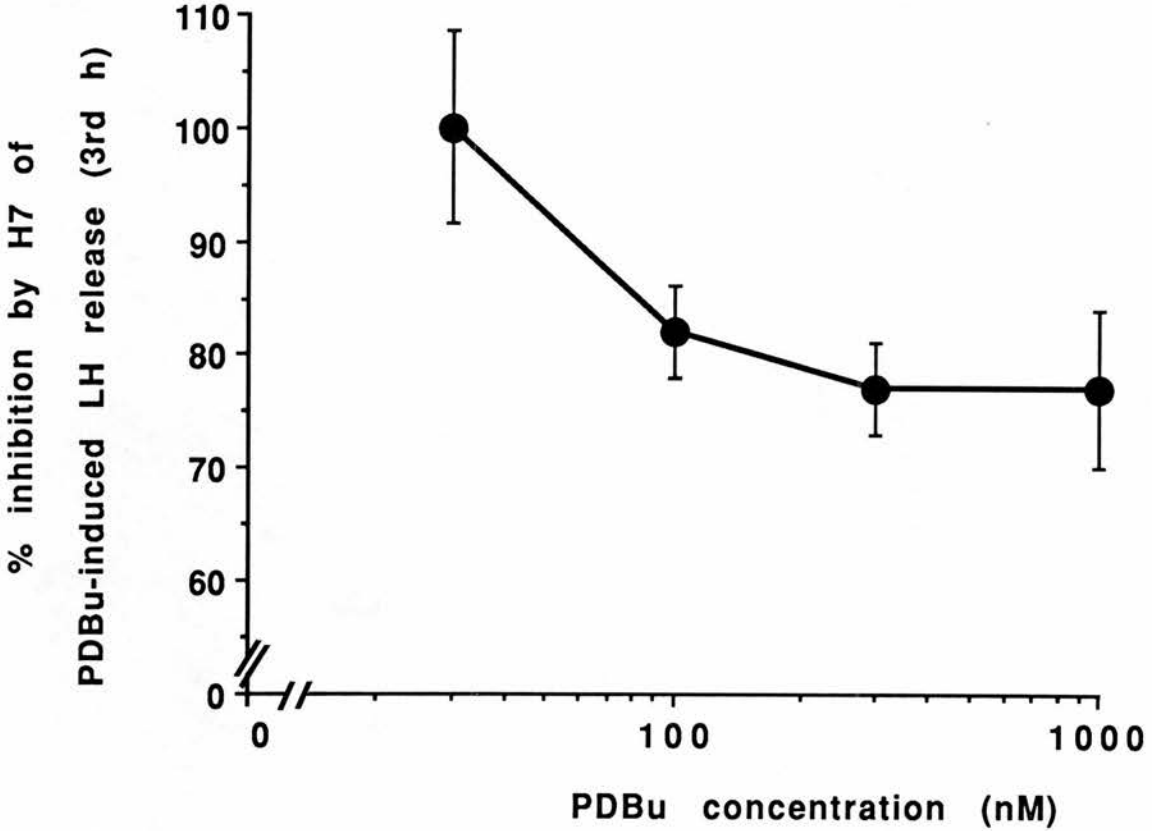

% inhibition by H7 of
PDBu-induced LH release (3rd h)
110
100
90
80
70
60
0
0
100
1000
PDBu concentration (nM)

TABLE 3.1

The effect of staurosporine on ionomycin-induced LH release from untreated and LHRH-pretreated pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated in vitro for 3 hours. In the basal hour there was medium alone (controls) or staurosporine (300 nM). In the 1st hour there was (in addition to staurosporine) ionomycin (50 μM, I_1). or LHRH (1 nM, L_1), followed by a 2nd h with ionomycin (I_2). The statistical significance of the inhibitory actions of staurosporine were determined (*$p < 0.05$). Values given are the mean ± SEM with the number of separate determinations in parentheses.

	LH release (fold of basal h)	
	1st h	**2nd h**
I_1 - I_2	19.2 ± 2.5 (7)	13.0 ±2.4 (7)
I_1 - I_2 + staurosporine	18.5 ± 3.0 (7)	10.5 ± 1.9 (7)
L_1 - I_2	3.7 ± 0.2 (6)	18.7 ± 2.5 (6)
L_1- I_2 + staurosporine	3.3 ± 0.7 (6)	*10.3 ± 0.9 (6)

TABLE 3.2

The effect of staurosporine on mezerein-induced LH and GH release from pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated *in vitro* for 4 hours. In the basal hour there was medium alone (baseline) or staurosporine (300 nM). In the following 3 consecutive hours the incubations contained the same and/or mezerein (300 nM). Values are for LH release measured in the 3rd h and GH release measured in the 1st h with mezerein. The statistical significance of the actions of staurosporine on mezerein-induced hormone release are shown as $*p < 0.05$. Values given are the mean ± SEM and the number of separate determinations are in parentheses.

	LH release (μg/l)	**GH release (μg/l)**
baseline	3.9 ± 0.4 (4)	480.7 ± 105.5 (4)
mezerein	39.1 ± 4.3 (5)	1820.3 ± 234.7 (5)
mezerein + staurosporine	*2.9 ± 0.4 (4)	*510.0 ± 38.7 (4)

TABLE 3.3

The effect of PKC inhibitors on 1,2-dioctanoyl-*sn*-glycerol (DOG)-induced LH release from pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated *in vitro* for 4 consecutive hours. In the basal hour, tissue was incubated in medium only (baseline), or with staurosporine (300 nM) or H7 (30 μM). In the following 3 consecutive hours in addition to these drugs, tissue was incubated with DOG (200 μM) as appropriate. Values are for LH release measured during the 3rd h of incubation with DOG with and without the appropriate PKC inhibitor. Net LH release was significantly enhanced above baseline levels in the presence of DOG ($^{\dagger}p < 0.05$). Staurosporine, but not H7, significantly inhibited DOG-induced LH release ($*p < 0.05$,) when compared to release in the presence of DOG only. Values are means ± SEM and the number of determinations are shown in parentheses.

	net LH release (μg/l)
baseline	3.6 ± 0.3 (5)
DOG	†8.5 ± 0.8 (6)
DOG + staurosporine	*2.4 ± 0.4 (6)
DOG + H7	7.7± 0.3 (6)

TABLE 3.4

The effect of PKC inhibitors on phorbol 12,13-dibutyrate-induced PKC activity partially purified from rat anterior pituitary cytosol

Phorbol 12,13-dibutyrate (1 μM)-induced histone IIIS kinase activity was measured in the presence of phosphatidylserine, with either the presence (100 μM free Ca^{2+}) or absence (> 3 nM free Ca^{2+}) of Ca^{2+}.

	IC_{50} values	
	staurosporine (nM)	**H7 (μM)**
Ca^{2+}-dependent	101 ± 39	17 ±4
Ca^{2+}-independent	171 ± 46	121 ± 18

CHAPTER 4

EVIDENCE FOR A ROLE OF PHOSPHOLIPASE A_2 IN LUTEINIZING HORMONE-RELEASING HORMONE RECEPTOR SIGNALLING IN RAT ANTERIOR PITUITARY TISSUE

4.1 INTRODUCTION

In Chapter 3, it was suggested that pharmacologically distinct forms of PKC or a PKC-like kinase may be involved in the induction of LHRH priming and PKC activator-induced LH release and GH release from pro-oestrous rat anterior pituitary tissue. Since the temporal patterns of phorbol ester-induced LH and GH release were different (Figure 3.5), the sequence of events that lead from PKC activation to hormone release in gonadotrophs is probably very different from that in somatotrophs. Thus, different PKC forms may induce LH and GH release and LHRH priming by phosphorylating different cellular targets. One potential target for the PKC(s) which induce LH release, and possibly those which mediate LHRH priming, may be phospholipase A_2 (PLA_2), or an intermediate factor which leads to the activation of PLA_2.

The release of LH can be induced from primary cultures of rat gonadotrophs by the PLA_2 activator, melittin, and by exogenously added snake venom PLA_2 (Kiesel *et al,* 1985). The actions of LHRH are accompanied by an increased release of AA and its metabolites (Vanderhoek *et al,* 1984; Catt *et al,* 1985) and exogenously added AA, and several AA metabolites, can induce LH release from dispersed anterior pituitary cell cultures (Naor and Catt, 1981; Naor *et al,* 1983; Hulting *et al,* 1984; Conte *et al,* 1986; Kiesel *et al,* 1987). Since these observations suggest that cellular responses to LHRH may involve PLA_2 activation, a possible role of PLA_2 in LH responses to LHRH and PKC activators was determined using pro-oestrous rat anterior pituitary tissue and the mouse gonadotroph-derived α T3-1 cell line.

4.2 MATERIALS AND METHODS

4.2.1 Hormone release measurements

Hemipituitaries were removed from adult female COB Wistar rats that had been anaesthetised and decapitated by 11.00 am on pro-oestrus and hormone release was measured using the methods described in section 2.2.3. The medium was radioimmunoassayed for LH, FSH and GH (Niswender *et al,* 1968; Daane and Parlow, 1971). The standards used were NIH-LH-S18 for the data in Figures 4.1, 4.2 and for some of the data in Figure 4.9 and Table 4.10 and NIADDK-rat LH-RP2 for the remaining figures and tables, NIADDK-rat-GH-RP-2 and NIADDK-rat FSH-RP2.

4.2.2 [^{3}H]-Arachidonic acid release measurements

[^{3}H]-Arachidonic acid release was measured from pre-labelled pro-oestrous anterior pituitary pieces and α T3-1 cells as described in section 2.2.5. After labelling with [^{3}H]-AA, pairs of pituitary quarters or 12 well plates of confluent α T3-1 cells were incubated for 15 min in the presence or absence of the appropriate drugs. The incubation medium was removed and the [^{3}H]-AA released was measured by lipid extraction and reverse-phase liquid chromatography on octadecyl silica (ODS) using the solvent system described by Powell (1982).

4.2.3 Drugs

Luteinizing hormone-releasing hormone, quinacrine dihydrochloride, aristolochic acid sodium salt, Ro 31-4493, Ro 31-4639, H7, cycloheximide and melittin were made up as stock solutions in distilled H_2O. Ionomycin, phorbol 12,13-dibutyrate (PDBu), 4-chloro-N-(*p*-pentylcinnamoyl) anthraniric acid (ONO-RS-082) RHC 80267, staurosporine, Ro 31-8220, nordihydroguaiaretic acid (NDGA), and indomethacin were made up as stock solutions in DMF. Arachidonic acid, arachidic acid, *p*-bromophenacyl bromide (BrPheBr), 7, 7-dimethyleicosadienoic acid (DEDA) and 4, 8, 11, 14-eicosatetraynoic acid (ETYA) were made up as stock solutions in

ethanol. The maximum concentration of DMF or ethanol (0.05% v/v) was used in control experiments and had no effect on either hormone release or [^{3}H]-AA release.

4.2.4 Data analysis

All statistical analysis was carried out using the Mann-Whitney U-test unless otherwise stated.

4.3 RESULTS

The effect of phospholipase A_2 inhibitors on LHRH responses measured in pro-oestrous rat hemipituitary pieces

Figure 4.1 shows the effect of the PLA_2 inhibitor, quinacrine (50 μM) (Markus and Ball, 1969; Löffler *et al,* 1985) on LH (Figure 4.1a) and FSH (Figure 4.1b) release from pro-oestrous rat hemipituitaries, measured over consecutive hourly incubations with LHRH (1 nM). Neither basal h, nor 1st h LHRH-induced LH release was significantly altered in the presence of quinacrine. However, 2nd h LHRH-induced LH release, where LHRH priming is expressed, was significantly inhibited by quinacrine (50 μM; $p < 0.05$) to levels that were 44.5 ± 3.1% of control. Similarly, 2nd h, but not basal h or 1st h, LHRH-induced FSH release was significantly inhibited by quinacrine (Figure 4.1b). Quinacrine, at concentrations up to 100 μM, caused a dose-dependent inhibition of 2nd h LHRH-induced LH release to levels approaching those measured during the 1st h of LHRH stimulation, but never to basal h levels (Figure 4.2a). Another PLA_2 inhibitor, ρ-bromophenacyl bromide (BrPheBr) (Drenth *et al,* 1976) also blocked LHRH-induced LH release from primed tissue to levels approaching those measured in the 1st h of LHRH incubation, without affecting 1st hour LH release (Figure 4.2b). However, at BrPheBr concentrations of 50 μM and above, basal h LH release was elevated above control levels, suggesting that this compound may have non-specific actions at high concentrations.

Other putative inhibitors of PLA_2 activity such as aristolochic acid (50 μM) (Vishwanath *et al,* 1988) and ONO-RS-082 (10 μM) (Banga *et al,* 1986) inhibited 2nd h LHRH-induced LH release without altering either basal h or 1st h LHRH-induced LH release. The inhibitory effects of these compounds on the 2nd, but not the 1st h of LHRH-induced LH release from pro-oestrous rat hemipituitaries indicate that PLA_2 activation may be required for LHRH priming, but not for initial responses to the peptide. However, other putative PLA_2 inhibitors, Ro 31-4493 (100 μM), Ro 31-4639 (100 μM) (Davis *et al,* 1988) and 7,7-dimethyleicosadienoic acid (DEDA) (30 μM) (Cohen *et al,* 1984) had no significant inhibitory effect on LHRH-induced LH release at any hour of incubation, when used at concentrations greater than those reported to cause 50% inhibition of PLA_2 activity (Table 4.1).

Neither quinacrine (100 μM), ONO-RS-082 (10 μM), aristolochic acid (150 μM), DEDA (30 μM), Ro 31-4493 (100 μM) nor Ro 31-4639 (100 μM) had any significant effect on baseline gonadotrophin release when measured over several hours of incubation.

Evidence that phospholipase A_2 activation is required specifically for the induction of LHRH priming in pro-oestrous rat anterior pituitary pieces

To examine the role of PLA_2 in LHRH priming in more detail, a number of experiments were carried out, similar to those which were used to ascertain the role of PKC in LHRH responses (see Chapter 3, section 3.3). A first experiment examined the effect of quinacrine on changes in pituitary responsiveness which occur during the 1st h of LHRH incubation (Figure 4.3a). Hemipituitary pieces were incubated for a basal h in medium only or with quinacrine (50 μM), followed by a 1st h incubation with, in addition, LHRH (1 nM). After the 1st h incubation, quinacrine was removed by washing the tissue three times in fresh pre-warmed and pre-gassed MEM and the washed tissue was incubated for a final hour (2nd h) with LHRH only. Control treatments received identical incubations with LHRH and washing, but in the

absence of quinacrine. Consistent with the results shown in Figures 4.1 and 4.2, quinacrine did not inhibit either basal h or 1st h LHRH-induced LH release (Figure 4.3a). However, following a 1st h incubation with LHRH and quinacrine, 2nd h LHRH-induced LH release was significantly reduced in comparison to the controls, although the tissue had been washed to remove the inhibitor from the 2nd h incubation period. These results indicate that quinacrine may inhibit the induction of LHRH priming, reducing the 2nd h response to LHRH as a consequence. However, it is possible that the washout procedure was not completely effective and that a significant amount of quinacrine remained in the tissue during the 2nd h of LHRH incubation. Consequently, additional experiments were carried out to further examine the effects of quinacrine on the induction LHRH priming.

In a second experiment, the effect of quinacrine was examined on LHRH-induced LH release from pituitary tissue which had been previously primed (Figure 4.3b). Hemipituitaries were incubated for a 1st h with LHRH followed by a further 2 consecutive hourly incubations with LHRH, either in the presence or absence of quinacrine (50 μM). Quinacrine had no significant inhibitory action on LHRH-induced LH release from pre-primed tissue, even over two hours of incubation which would allow for any possible slow-developing inhibitory effect on the mechanism of hormone release.

To test the action of quinacrine on the secretory apparatus of the gonadotroph, the effect of this drug was examined on LH release induced by raising intracellular Ca^{2+} levels (Table 4.2). In the presence of ionomycin (30 μM), LH release was significantly increased ($p < 0.05$, Mann-Whitney U-test) above basal h levels, but with a temporal pattern that did not exhibit 'priming'. Quinacrine (50 μM) had no significant inhibitory effect on ionomycin-induced LH release measured during any hour of incubation indicating that the inhibitor did not alter the general Ca^{2+}-induced secretory apparatus of the gonadotroph. In addition, these results suggest that PLA_2 is not involved in the mechanism of Ca^{2+}-induced LH release. In

contrast, ionomycin-induced LH release from tissue which had been previously primed by a 1st h incubation with LHRH was significantly inhibited when quinacrine was present during the LHRH incubation period. This is consistent with the idea that, although priming relates specifically to a cascade of intracellular signals elicited by LHRH, the consequential facilitation of release to any given stimulus in primed tissue applies not only to LHRH as a secretagogue but to other general post-priming stimuli, such as Ca^{2+} ionophore (Pickering and Fink, 1979).

In summary, PLA_2 inhibitors blocked LHRH responses only when present during the time when LHRH priming takes place, i.e. the 1st h of LHRH treatment (Figures 4.1, 4.2, 4.3 and Table 4.1, 4.2). In addition, quinacrine did not inhibit the actions of secretagogues that do not evoke a priming response. These results, therefore, indicate an involvement of PLA_2 in the induction, but not in the expression of LHRH priming.

Is an H7-resistant form of PKC involved in the mechanism of phospholipase A_2 activation during LHRH priming?

The analogous effects of PKC inhibitors and PLA_2 inhibitors on LHRH priming imply a possible connection between PKC and PLA_2 activation in the induction of this response. In one possible sequence of events, AA produced by PLA_2 action may directly activate PKC (section 1.2.2 and 1.3.5). To test this hypothesis, the effect of PKC inhibitors was examined on secretory responses to AA. Arachidonic acid (300 μM) could induce a significant increase in LH release during the 2nd and 3rd h of incubation with the fatty acid (Figure 4.4a). However, neither staurosporine nor H7 had any significant inhibitory effect on AA (300 μM)-induced LH release (Table 4.3) suggesting that AA does not induce LH release by a mechanism that involves PKC. Furthermore, the saturated congener of AA, arachidic acid (300 μM), which lacks many of the biological actions of AA (Beaumier *et al,* 1987; Negishi *et al,* 1990), could also induce a significant increase in LH release

during the 2nd and 3rd h of incubation (Figure 4.4b). It would seem, therefore, that the direct actions of AA on LH release may be non-specific.

In an alternative sequence of events, PKC activation may modulate PLA_2 activity. To test this possibility, the effect of PLA_2 inhibitors was examined on PDBu-induced LH release and as a contrast, PDBu-induced GH release (Figure 4.5 and Table 4.4). Phorbol 12,13-dibutyrate (300 nM)-induced LH release was dose-dependently inhibited by quinacrine ($IC_{50} = 20 \pm 9$ μM). In contrast, PDBu-induced GH release measured was unaltered by quinacrine (Figure 4.5b), even at concentrations as high as 100 μM where PDBu-induced LH release was decreased to levels which were approximately 10% of control. Aristolochic acid (100 μM) and BrPheBr (50 μM) also inhibited PDBu-induced LH release but not PDBu-induced GH release (Table 4.4). The actions of these inhibitors suggest that PLA_2 activation may be involved in the mechanism by which the PKC(s) induces LH release, but not GH release.

The influence of LHRH on gonadotroph PLA_2 activity was examined, more directly, by measuring [^{3}H]-AA release from pre-labelled anterior pituitary tissue. After a 15 min incubation with LHRH (1 nM), medium [^{3}H]-AA levels increased by approximately 2-fold over basal levels (Table 4.5). To determine the pathway of LHRH-induced [^{3}H]-AA release, the effects of quinacrine and the DAG lipase inhibitor, RHC 80267 were examined. These inhibitors were used at concentrations reported to inhibit the activity of their target enzymes with minimal side effects (Hoffman *et al,* 1982; Sutherland and Amin, 1982). [^{3}H]-Arachidonic acid release induced by LHRH was inhibited by quinacrine (50 μM), but not by RHC 80267 (80 μM) (Table 4.5), indicating that the main route of [^{3}H]-AA release induced by LHRH is via a pathway involving PLA_2.

To test for the possible involvement of the H7-resistant PKC-like priming kinase in the mechanism of LHRH-induced activation of PLA_2, the effects of H7 and staurosporine were examined on LHRH-induced [^{3}H]-AA release (Table 4.6). [^{3}H]-

Arachidonic acid release induced by LHRH was readily inhibited by staurosporine (300 nM), a concentration which similarly inhibits LHRH priming (Chapter 3), to levels that were not significantly different from basal. However, LHRH-induced [^{3}H]-AA release was relatively resistant to inhibition by H7 at a concentration (30 μM) which is unable to significantly inhibit LHRH priming (Chapter 3) (Table 4.6). Thus, during LHRH action on the gonadotroph, the action of an H7-resistant form of PKC may be instrumental in increasing PLA_2 activity, an event which may participate in bringing about a primed state.

The pharmacology of the [^{3}H]-AA release response, with respect to PKC inhibitors, was analysed further. Phorbol 12,13-dibutyrate (300 nM) induced a dose-dependent increase in [^{3}H]-AA release from pre-labelled pro-oestrous rat anterior pituitary tissue (Figure 4.6), a response which was readily inhibited by quinacrine (50 μM) but not RHC 80267 (80 μM) (Chapter 2, Table 2.2). This result is consistent with the hypothesis that PKC may induce [^{3}H]-AA release by modulating anterior pituitary PLA_2 activity, rather than by other possible routes of free AA production. Both staurosporine and Ro 31-8220 completely inhibited 300 nM PDBu-induced [^{3}H]-AA release from pre-labelled tissue with IC_{50} values of 28.4 ± 17.9 nM and 5.1 ± 7.8 μM respectively (Figure 4.7a and 4.7b). Phorbol ester-induced [^{3}H]-AA release was also inhibited by H7, but in a biphasic manner (Figure 4.7c). Although more than 50% of the response to PDBu was blocked by H7 concentrations as little as 1 μM, no further inhibition was observed until the H7 concentration was then further increased by over 30-fold, suggesting that the PKC form(s) mediating over 40% of the response was highly resistant to H7. Thus, although H7-resistant forms of PKC are involved in LHRH-induced modulation of PLA_2 activity, both H7-resistant and -sensitive forms may be involved in the mechanism of PDBu-induced [^{3}H]-AA release. Neither staurosporine (300 nM), H7 (100 μM), Ro 31-8220 (30 μM), quinacrine (50 μM) nor RHC 80267 (200 μM) had any significant effect on baseline [^{3}H]-AA release (Table 2.1).

The priming effect of LHRH measured in pro-oestrous rats *in vivo* and *in vitro,* is dependent upon protein synthesis (Pickering and Fink, 1976; Debeljuk *et al,* 1978). It is unlikely that this protein synthesis-dependent step represents the synthesis of new LH as there is no change in total pituitary LH content during priming (Pickering and Fink, 1979; Speight and Fink, 1981). The relationship of protein synthesis to PKC and to PLA_2 activation in the mechanism of LHRH priming was therefore investigated. The protein synthesis inhibitor, cycloheximide (Obrig *et al,* 1971), at a concentration of 50 μM, significantly inhibited PDBu (300 nM)-induced LH release, but not GH release during all hours of incubation (Figure 4.8). Cycloheximide also inhibited LHRH- and PDBu-induced [^{3}H]-AA release (Table 4.6). In contrast, cycloheximide had no effect on LH release induced by the PLA_2 activator, melittin (Habermann, 1972), during any hour of incubation (Figure 4.9). Thus, it appears that in the LHRH receptor signal transduction system, protein synthesis may be required for a process following PKC activation, but prior to the action of PLA_2. These results are consistent with a model of LHRH receptor signalling in which PKC modulates PLA_2 activity, in a protein synthesis-dependent manner, to induce priming.

Arachidonic acid as a possible mediator of LHRH priming

Since PLA_2 activation may be required for the induction of LHRH priming, and the actions of LHRH are accompanied by an increase in free [^{3}H]-AA levels, AA may be a mediator of the priming effect. As shown earlier (Figure 4.4), AA could induce LH release from pro-oestrous hemipituitaries but probably by a non-specific effect. Since PLA_2 activation is important for the induction of priming, but not for LH release, a more probable action of AA may be to alter gonadotroph responsiveness. To test the actions of AA on gonadotroph responsiveness, pro-oestrous rat hemipituitaries were pre-incubated for 1 h with AA (300 μM), or arachidic acid (300 μM), followed by 2 further consecutive hourly incubations (1st h,

2nd h) with LHRH only (1 nM) (Table 4.8). To allow for the direct effects of AA and arachidic acid on LH release, control treatments contained the appropriate fatty acid in the basal h, but were incubated in medium only during the 1st and 2nd h. Hormone release measured throughout these control incubations was subtracted from values obtained in the presence of LHRH. Prior incubation with AA, but not arachidic acid, significantly enhanced net LHRH-induced LH release measured over 2 consecutive hours ($p < 0.05$) (Table 4.8). Since AA treatment could increase pituitary responsiveness to LHRH, analogous to a priming effect, in a manner that was distinct from its non-specific actions on gonadotrophin release, it seems possible that the generation of AA may contribute to bringing about priming.

The effect of inhibitors of arachidonic acid metabolism on LHRH responses measured in pro-oestrous rat hemipituitaries

Since previous reports have suggested that metabolites of AA may modulate gonadotrophin release (Naor *et al,* 1983; Snyder *et al,* 1983; Catt *et al,* 1985; Kiesel *et al,* 1987), a possible role for AA metabolites in LHRH responses in pro-oestrous rat hemipituitary pieces was examined using a number of inhibitors of the pathways of AA metabolism. At a concentration of 10 μM, where 4,8,11,14-eicosatetraynoic acid (ETYA) is reported to inhibit the lipoxygenase and cyclo-oxygenase pathways of AA metabolism (Hamberg and Samuelson, 1974), LHRH-induced LH release was unaltered during any hour of incubation (Table 4.9) suggesting that neither lipoxygenase nor cyclo-oxygenase metabolites are required for LHRH responses in pro-oestrous rat hemipituitaries. However, ETYA, at a concentration of 30 μM, inhibited 2nd h, but not 1st h, LHRH-induced LH release to levels that were not significantly different from basal h measurements. Although ETYA can inhibit the epoxygenase pathway of AA metabolism at this higher concentration (Capdevilla *et al,* 1988), it can also block PLA_2 activity (Lanni and Becker, 1985). Nordihydroguiaretic acid (NDGA), when used at a concentration (10

μM) reported to inhibit the epoxygenase and lipoxygenase pathways (Smith and Bowman, 1982; Capdevilla *et al,* 1988), had no effect on LHRH responses during any hour (Table 4.9). However, when used at a concentration of 30 μM, NDGA inhibited both 1st h and 2nd h LHRH-induced LH release to baseline levels, but at this level NDGA can also inhibit PLA_2 activity (Lanni and Becker, 1985). The cyclo-oxygenase inhibitor, indomethacin, had no effect on LHRH-induced LH release during any hour (Figure 4.10) when used at concentrations up to 30 μM. However, 300 μM indomethacin inhibited 2nd h LHRH-induced LH release to basal h levels of release, suggesting that this inhibitor may have a slow-developing toxic effect on hormone release at high concentrations.

Neither ETYA (10 μM), NDGA (30 μM) nor indomethacin (30 μM) had any significant effect on baseline LH release measured over several hours of incubation.

The involvement of phospholipase A_2 in LHRH receptor signalling in α T3-1 cells

The anterior pituitary gland consists of a heterogeneous cell population, only around 10% of which are gonadotrophs. It is therefore difficult to determine the contribution of this small population of cells in responses measured in whole pituitary. A clonal cell line has recently been developed from anterior pituitary tumours generated in transgenic mice (Windle *et al,* 1990), and in the following experiments, this α T3-1 cell line was used to further investigate and compare the effects of phorbol esters and LHRH on gonadotroph PLA_2 activity. When [^{3}H]-AA pre-labelled α T3-1 cells were incubated with LHRH for 15 min, there was a significant increase in [^{3}H]-AA release (Table 4.10). However, LHRH-induced AA release from α T3-1 cells was not inhibited by either staurosporine (300 nM) or H7 (30 μM). Furthermore, PDBu (300 nM) did not induce any significant release of [^{3}H]-AA from α T3-1 cells. The effects of PDBu and PKC inhibitors on LHRH responses in α T3-1 cells contrast strongly with their actions on pro-oestrous rat

pituitary [^{3}H]-AA release (Table 4.6 and Figure 4.6), suggesting that quite different cellular signalling pathways may be involved in these transformed cells, limiting their usefulness as a model of gonadotrophs *in vivo*.

4.4 DISCUSSION

Previous reports suggest that stimulation of rat gonadotroph and rat granulosa cell LHRH receptors can lead to activation of PLA_2 (Naor and Catt, 1981; Minegishi *et al,* 1987). The present evidence suggests that, in pro-oestrous rat hemipituitary pieces, activation of PLA_2 may be required for the induction of LHRH priming, but not for initial LHRH-induced gonadotrophin release. Quinacrine, BrPheBr, aristolochic acid and ONO-RS-082 blocked 2nd h LHRH-induced release of primed LH, but not 1st h LHRH-induced LH release (Figures 4.1, 4.2 and Table 4.1), with potencies similar to their effects on PLA_2-mediated responses in other cell types (Löffler *et al,* 1985; Banga *et al,* 1986; Rosenthal *et al,* 1989; Tohmatsu *et al,* 1989). Notably, the concentration of quinacrine which was reported to inhibit long-term secretory responses to LHRH in primary cultures of rat anterior pituitary cells (IC_{50} = 20 μM) (Naor and Catt, 1981) is within the range described here for the inhibition of the LHRH priming (IC_{50} = 34.0 ± 10.3 μM). Using pro-oestrous rat tissue, the 2nd h response to LHRH was reduced by these inhibitors to levels that approached 1st h levels of LHRH-induced LH release, but never to baseline levels, suggesting that PLA_2 activation may be required for enhanced gonadotroph responsiveness. Consistent with this hypothesis, LHRH-induced LH release was inhibited only if quinacrine was present throughout the time when LHRH priming occurred, i.e. during the 1st h of LHRH incubation, whereas LHRH-induced LH release from pre-primed tissue was not blocked by quinacrine (Figure 4.3). Therefore, the induction, but not the expression, of priming may well be dependent upon PLA_2 activation. Quinacrine did not appear to inhibit the release of LH from previously primed tissue by affecting the general mechanism of gonadotrophin release

since this drug did not alter ionomycin-induced LH release. However, if the tissue was primed by exposure to LHRH, facilitated LH release in response to Ca^{2+}-ionophores was blocked by quinacrine to 'non-primed' levels (Table 4.2). Interestingly, long-term secretory responses to LHRH measured in dispersed rat anterior pituitary cells, which do not exhibit an obvious priming response, are also dependent upon PLA_2 action (Naor and Catt, 1981). It seems, therefore, that although PLA_2 activation is involved in the later stages of hormone release in both the dispersed cell and intact tissue models of LHRH action, some other factor may limit the expression of priming in dispersed cells. For example, the products of PLA_2 action may have paracrine effects on adjacent cells *in situ*, an action which will be altered by the process of cell dispersal.

The specificity of both quinacrine and BrPheBr as inhibitors of PLA_2 action has been questioned in a number of studies (Irvine, 1982; Dise *et al,* 1982; Chang *et al,* 1987a). Quinacrine may inhibit PLA_2 activity by interacting with the hydrophobic regions of the phospholipid substrate and, at high concentrations, may perturb membrane architecture and alter cell function (Dise *et al,* 1982). However, quinacrine did not alter the LH release induced by elevating intracellular Ca^{2+} levels through the use of iomomycin (Table 4.2), suggesting that a non-specific action of this drug is unlikely to be responsible for its effects on LHRH responses. Furthermore, quinacrine attenuated PDBu-induced LH release without any significant effect on corresponding GH release (Figure 4.5). Bromophenacyl bromide inhibited PLA_2 activity by forming a covalent bond with a histidine residue which is situated near the Ca^{2+}-binding site of the enzyme (Volwerk *et al,* 1974; Drenth *et al,* 1976; Roberts *et al,* 1977), but can also alkylate thiol and amine groups of many different proteins (Erlanger *et al,* 1965) which may account for the effects of high concentrations of BrPheBr on baseline LH release. Although BrPheBr and quinacrine are putative inhibitors of PLC activity (Hofmann *et al,* 1982), these compounds were unable to prevent the presumed PLC-dependent, 1st h LHRH-induced LH release. In

addition, at the concentrations used here, these compounds have been reported to inhibit PLA_2 in other systems without affecting PLC activity (Lazarewicz *et al,* 1988). Furthermore, ONO-RS-082, which can inhibit PLA_2 activity in platelets without affecting PLC activity (Tohmatsu *et al,* 1989; Banga *et al,* 1986), blocked the primed response to LHRH.

Although the LHRH priming phenomenon was inhibited by quinacrine, BrPheBr, aristolochic acid and ONO-RS-082, a number of other putative PLA_2 inhibitors (DEDA, Ro 31-4493 and Ro 31-4639) had no effect on LH release measured over any hour of incubation with LHRH (Table 4.1). Other workers have shown that DEDA can inhibit PLA_2 activity *in vitro*, but not *in vivo*, and have suggested that the efficacy of DEDA as an inhibitor *in vitro* may relate to the characteristics of the enzyme in isolation (Cohen *et al,* 1984). Both Ro 31-4493 and Ro 31-4639 can block PLA_2 action in intact cells with an IC_{50} value of 10 - 25 μM (Roldan and Mollinedo, 1991; Henderson *et al,* 1989). In certain cell types, therefore, these compounds do appear to gain access to the cell interior although it is not certain whether all cell types are readily permeable to these compounds (Roche Products plc, unpublished observations). Both Ro PLA_2 inhibitors were designed to interact with polar amino acid side chains in the active site of porcine pancreatic PLA_2 (Davis *et al,* 1988). However, this enzyme has no detectable sequence homology with cytosolic (presumably hormonally-regulated) PLA_2s (Clark *et al,* 1990, 1991b). As a consequence, both compounds may be weak inhibitors of the PLA_2(s) that has a role in LHRH receptor signal transduction. In agreement with both of these hypotheses regarding the failure of the Ro compounds to inhibit priming, experiments from our laboratory have shown that quinacrine, but neither Ro 31-4493 nor Ro 31-4639 can inhibit PDBu-induced [^{3}H]-AA release from pro-oestrous rat anterior pituitary tissue ($87 \pm 7\%$ and $90 \pm 4\%$ of the control response to PDBu in the presence of Ro 31-4493 and Ro 31-4639, respectively). Pharmacological differences between enzymes isolated from different tissue sources have been reported previously (Löffler *et al,*

1985; Nixon *et al,* 1985). For example, another PLA_2 inhibitor, WY-48, 489, readily inhibits membrane-associated PLA_2 isolated from human platelets but has little effect on pancreatic PLA_2 activity (Marshall and Chang, 1990). It is possible that inhibitors which display selectivity for different forms of PLA_2 may be developed as therapeutic agents in the future.

In agreement with the pharmacological evidence presented here which suggests that activation of LHRH receptors modulate gonadotroph PLA_2 activity, LHRH induces a relatively rapid (15 min) increase in a quinacrine-sensitive, but RHC 80267-insensitive release of [^{3}H]-AA from pro-oestrous rat anterior pituitary pieces (Table 4.5). Unfortunately, due to the small population of gonadotrophs in anterior pituitary tissue, it was not possible to measure the cellular content of [^{3}H]-AA after stimulation. The exact relationship between medium [^{3}H]-AA levels (as measured in these experiments) and cellular-free AA is therefore unclear. Although a time-course of LHRH-induced [^{3}H]-AA release from pro-oestrous rat tissue was not studied in detail, in dispersed pituitary cells LHRH induces a significant release of AA by 10 minutes of treatment which gradually increases over a period of an hour (Naor and Catt, 1981), and this correlates with the time-course of the development of priming *in vitro* (Pickering and Fink, 1976). However, in contrast with these results, a previous report has shown that RHC 80267, at concentrations that block DAG lipase activity with little or no effect on PLA_2, inhibited approximately 40% of LHRH-induced LH release from dispersed anterior pituitary cells (Chang *et al,* 1988). The reason why DAG lipase is apparently involved in LHRH responses in dispersed cells but not in pro-oestrous rat pituitary tissue is uncertain, but may reflect an alteration in gonadotroph function which occurs following cell dispersal and culture (see section 1.4.3).

The involvement of PLA_2 in LHRH responses in pro-oestrous rat anterior pituitary tissue may have been further demonstrated by the generation of lysophospholipid following agonist stimulation. However, it is now clear that certain

cytosolic PLA_2 forms exhibit lysophospholipase activity (Leslie, 1991). Therefore, the absence of lysophospholipid production in LHRH-stimulated cells may not indicate conclusively that PLA_2 is not involved in agonist-induced AA release. Alternatively, following LHRH treatment, changes in anterior pituitary PLA_2 activity may have been determined by measuring partially-purified PLA_2 activity in a cell-free assay using either a fluorogenic or radiolabelled PLA_2 substrate (Clark *et al,* 1990; Gronich *et al,* 1990; Piomelli and Greengard, 1991). However, since gonadotrophs are a relatively small proportion (~10%) of the cell types in anterior pituitary tissue, any change in gonadotroph PLA_2 activity may be difficult to detect using this method. Although changes in α T3-1 cell PLA_2 activity may be more readily detected in cell free assays, it is clear that LHRH-induced [^{3}H]-AA may be under different intracellular control in this clonal cell line than responses to LHRH measured in pro-oestrous rat anterior pituitary tissue (Figure 4.6, Tables 4.6 and 4.10). Thus, it is uncertain as to how stimulus-induced changes in α T3-1 cell PLA_2 activity will relate to changes in PLA_2 activity determined in pro-oestrous rat anterior pituitary tissue.

During the LHRH receptor signal, modulation of gonadotroph PLA_2 activity may be dependent upon the activity of a PKC-like kinase, suggesting that PLA_2 activation may occur down-stream from the action of PLC which, presumably, was the origin of the DAG which led to PKC activation. An initial indication of a possible link between PLA_2 and PKC activation came from the comparable actions of PLA_2 inhibitors (quinacrine, BrPheBr, ONO-RS-082 and aristolochic acid) and PKC inhibitors (staurosporine, K252a, Ro 31-8220 and H7) on the induction of the priming effect (see also Chapter 3). In addition, staurosporine and H7 inhibited LHRH-induced [^{3}H]-AA release at concentrations which are equivalent to their effects on LHRH priming (Figures 3.2 and 3.3 and Table 4.5). Thus, LHRH receptor-mediated activation of an H7-resistant, PKC-like kinase may participate in modulating PLA_2 activity and ultimately result in priming. In agreement with this sequence of events,

PLA_2 inhibitors blocked PDBu-induced LH release (Figure 4.5) and PDBu treatment enhanced [^{3}H]-AA release (Figure 4.6). Although PKC-dependent phorbol ester-modulation of PLA_2 activity has been described in other cell types (Hartung and Toyka, 1987; Parker *et al,* 1987), there are some reports that phorbol esters may directly activate phospholipases (Billah *et al,* 1989; Billah and Anthes, 1990). It is unlikely that direct activation of PLA_2 can account for the effects of PDBu on anterior pituitary [^{3}H]-AA release since this effect required protein synthesis (Table 4.7), implying that the mechanism of PLA_2 activation is complex. Furthermore, PDBu-induced [^{3}H]-AA release from hemipituitaries was readily inhibited by staurosporine and Ro 31-8220 (Figure 4.7), indicating the involvement of PKC in this response. Phorbol 12,13-dibutyrate-induced [^{3}H]-AA release was inhibited by H7 in a biphasic manner suggesting that, in addition to a set of relatively H7-resistant PDBu-activated kinases, which presumably modulate PLA_2 activity in LHRH receptor signalling, a set of more conventional H7-sensitive PKC(s) are capable of activating PLA_2. These H7-sensitive PKC(s) which control PLA_2 activity may be involved in some function of the gonadotroph, other than priming, or may have a function in the control of AA release from other anterior pituitary cell types.

Modulation of PLA_2 activity by PKC requires protein synthesis and it seems quite possible that this process may represent the protein synthesis-dependent step in the LHRH priming response. Luteinizing hormone-releasing hormone-induced [^{3}H]-AA release, PDBu-induced [^{3}H]-AA release and PDBu-induced LH release were very markedly inhibited by cycloheximide (Table 4.7 and Figure 4.8). Therefore, protein synthesis may occur subsequent to PKC activation, consistent with a previous observation (Bourne *et al,* 1989). Melittin-induced LH release was not affected by cycloheximide (Figure 4.9), indicating that the processes beyond PLA_2 activation do not require protein synthesis. However, melittin can cause cell lysis and induce voltage-dependent ion conductances in planar lipid bilayers (Tosteson and Tosteson, 1981; Metz, 1986), thus melittin-induced LH release may not occur by an

entirely PLA_2-dependent process. Nevertheless, these results do suggest that PKC modulates PLA_2 activity in a protein synthesis-dependent manner. In smooth muscle and epithelial cells, PKC can regulate the rapid synthesis of an endogenous, melittin-like protein which activates PLA_2 (Clark *et al,* 1987a, 1991b) This PLA_2 activating protein (PLAP) has been shown to have a role in the leukotriene D_4 receptor signalling system (see Crooke *et al,* 1989 for review). Preliminary evidence obtained by Northern blot analysis has indicated that LHRH may modulate PLAP mRNA levels in pro-oestrous rat anterior pituitary tissue (Lutz and Mitchell, unpublished observations). Indeed, during LHRH receptor signalling, PKC-induced synthesis of a PLAP and subsequent PLA_2 activation could represent an appropriate protein synthesis-dependent mechanism by which LHRH priming may occur. Further investigations are required to examine the involvement of PKC in the control of PLAP expression by LHRH, perhaps by studying the effects of PKC inhibitors on LHRH induction of PLAP mRNA by Northern blotting, and on PLAP expression, using PLAP-selective antibodies.

Of the potential products of PLA_2-catalysed phospholipid hydrolysis, AA, may be a mediator of priming. Both AA and its biologically inactive congener, arachidic acid, could induce LH release, but only AA could enhance pituitary responsiveness (Table 4.8). Arachidic acid lacks many of the cellular actions of AA, for example, AA, but not arachidic acid, can induce Ca^{2+} release from intracellular stores (Beaumier *et al,* 1987) and can induce inositol phospholipid hydrolysis (Negishi *et al,* 1990). It is possible, therefore, that AA and arachidic acid may induce LH release by having a non-specific detergent-like action on the gonadotroph cell membrane (Karnovsky *et al,* 1982; Kaye *et al,* 1992). In dispersed anterior pituitary cells, LH release in response to AA and LHRH are additive (Chang *et al,* 1987b), suggesting that the mechanism by which AA induces LH release is separate from the mode of action of LHRH. Certainly, AA, at concentrations higher than those used here, can cause lysis of dispersed anterior pituitary cells (Liu and Jackson, 1989).

However, 300 μM AA, as used here, was unable to induce a significant increase in GH release from rat anterior pituitary pieces when measured over several hours of incubation, suggesting that a lytic effect of AA is unlikely to account for the actions of this fatty acid on LH release responses shown here. In addition, AA is readily metabolised (Needleman *et al,* 1986) and can be re-incorporated into cellular phospholipids (Irvine, 1982) and at high concentrations, AA can assume a miceller form in an aqueous environment (MacEwan *et al,* 1991), so it is likely that the free concentration of AA is considerably less than the empirical value. In other cell types, endogenous AA production can be dissociated from exocytosis (Churcher *et al,* 1990; Morgan and Burgoyne, 1990; Cockcroft, 1991) suggesting that AA may not be a direct mediator of secretion. This is probably also true in gonadotrophs where PLA_2 activation and AA production are not required for LH release, but for enhancing gonadotroph responsiveness. This effect of AA on gonadotroph responsiveness was not mimicked by arachidic acid, suggesting that the effect of AA was probably not due to a non-specific action of the fatty acid. However, AA was unable to completely mimic the degree of priming induced by LHRH and an additional increment in LHRH-dependent priming occurred following AA incubation, suggesting that AA may not be the sole mediator of priming. Lyso-phospholipid, the co-product of PLA_2 action, can have a number of biological actions and can be further metabolised to 1-alkyl-2 (R)-acetyl-glycerol-3-phosphorylcholine, (platelet-activating factor (PAF)). Platelet-activating factor can act on specific membrane bound receptors and, itself, can induce inositol phospholipid turnover, having either a paracrine effect on other cells in the anterior pituitary or an autocrine action on the cell from which it was released (see Shukla, 1991 for review). A possible role of both PAF and lysophospholipid as mediators of LHRH priming requires further investigation, perhaps by studying the effect of metabolically-stable species of PAF on gonadotroph responsiveness and of PAF receptor antagonists on LHRH responses. Other intracellular factors, such as raised Ca^{2+} levels, may contribute by acting in parallel,

or synergistically, with AA to achieve a maximal 'primed' secretory response. Indeed, in cell-free systems, AA can enhance fusion of secretory granules into membranes, in a Ca^{2+}-dependent manner, modulating exocytosis (Creutz, 1981). An equivalent action of AA in gonadotrophs may enhance Ca^{2+}-induced LH release.

The LHRH priming affect may not be dependent upon AA metabolism since neither ETYA nor NDGA had any effect on LHRH responses when either compound was used at a concentration (10 μM) which is reported to inhibit lipoxygenase, cyclo-oxygenase and epoxygenase activity (Hamberg and Samuelson, 1984; Smith and Bowman, 1981; Capdevilla *et al,* 1988) (Table 4.9). At a higher concentration (30 μM), ETYA and NDGA did inhibit priming, but at this concentration, both drugs also block PLA_2 activity (Lanni and Becker, 1985) so no clear interpretation can be made regarding these results. Indomethacin, did not alter LHRH-responses except at high, non-specific concentrations (Franson *et al,* 1980; Snyder *et al,* 1983) (Figure 4.10), consistent with previous reports which suggest that cyclo-oxygenase products do not have a role in LHRH receptor signalling (Naor *et al,* 1975a; Sundberg *et al,* 1975; Naor and Catt, 1981; Catt *et al,* 1985). Therefore, we could find no evidence to suggest that AA metabolites have a role in LHRH responses in pro-oestrous rat anterior pituitary tissue. However, both ETYA and NDGA can inhibit LHRH-induced LH release from primary cultures of anterior pituitary cells (Naor *et al,* 1983; Catt *et al,* 1985) suggesting a role for lipoxygenase metabolites in this system. In some studies, however, high, possibly non-specific concentrations of these inhibitors were used (Chang *et al,* 1986a, 1987b), so no clear conclusions can be made regarding these observations. Studies into the actions of several different lipoxygenase metabolites on LH release have produced conflicting results (section 1.4.4) and as a consequence, a distinct conclusion as to the role of specific leukotrienes as mediators in LHRH responses cannot be made. Nevertheless, selective leukotriene receptor antagonists can partially inhibit LHRH-induced LH release from primary cultures (Catt *et al,* 1985; Kiesel *et al,* 1991; Bito *et al,* 1992).

It is possible that these discrepancies may be accounted for by differences in the model of pituitary function chosen in each study (see Conn *et al,* 1992 for review) and that lipoxygenase products have a different function in LHRH receptor-signalling in pro-oestrous rat pituitary pieces than in dispersed pituitary cells that have been maintained in culture for several days. A role for leukotrienes as hypothalamic transmitters which are co-released with LHRH has been suggested (Lindgren *et al,* 1984). However, the physiological relevance of this possible hormonal action of leukotrienes has yet to be determined.

Epoxygenase metabolites, including 5, 6-epoxyeicosatrienoic acid, have been shown to induce LH release from primary cultures of anterior pituitary cells. However, these effects on LH release only occur at concentrations that are unlikely to be physiologically relevant (> 1 μM) (Snyder *et al,* 1983), and may reflect the ability of epoxygenase metabolites to act as Ca^{2+} ionophores (Snyder *et al,* 1986). Further studies using new, highly selective inhibitors of epoxygenase and lipoxygenase enzymes will help to clarify the role of these pathways of AA metabolism in LHRH responses in pro-oestrous rat anterior pituitary tissue.

Luteinizing hormone-releasing hormone induced release of [^{3}H]-AA from the male mouse gonadotroph α T3-1 cell line (Table 4.10), consistent with a role for PLA_2 in LHRH receptor signalling in these cells. However, in contrast to the results observed using pro-oestrous hemipituitaries, PDBu did not induce any significant release of [^{3}H]-AA from α T3-1 cells and PKC inhibitors were unable to block LHRH-induced LH release (Table 4.10). These results are most interesting since they suggest that LHRH receptors in α T3-1 cells may be coupled to different intracellular mechanisms than those which are linked to LHRH receptors in pro-oestrous rat gonadotrophs. Stimulation of α T3-1 cells with LHRH causes a number of intracellular changes that also occur in rat primary cell cultures and anterior pituitary pieces, including the production of inositol phosphates, PKC translocation, enhancement of voltage-sensitive Ca^{2+}-channel activity, an increase in gonadotrophin

α-subunit mRNA levels and PLD stimulation (Horn *et al,* 1991; Netiv *et al,* 1991). However, these cells do not express the β-gonadotrophin subunit (Windle *et al,* 1990) indicating that some functions of these cells are abnormal. The different responses of α T3-1 cells in comparison to pro-oestrous rat hemipituitaries may, in part, be due to the abnormal functioning of these cells, species differences and/or the differences in steroid influences. Certainly, dispersed anterior pituitary cells from pro-oestrous rats do not release [^{3}H]-AA in response to PDBu unless they have been treated with oestrogen (see Chapter 5), suggesting that steroids are required to maintain the pathway by which PKC activation leads to modulation of PLA_2 activity. Nevertheless, in contrast to its actions on pro-oestrous rat hemipituitaries, LHRH does not appear to modulate [^{3}H]-AA release from α T3-1 cells in a PKC-dependent manner. One possibility is that, in α T3-1 cells, LHRH activates PLA_2 by a mechanism which is independent of PLC-mediated inositol phospholipid turnover and PKC activation, perhaps by direct G-protein coupling of the LHRH receptor to PLA_2. Experiments examining the effect of the PLC inhibitor, neomycin, on LHRH-induced [^{3}H]-AA release from α T3-1 cells will help clarify the possible involvement of a PLC-independent pathway of [^{3}H]-AA release in these cells. Alternatively, LHRH may induce [^{3}H]-AA release from α T3-1 cells by a PLA_2-independent mechanism, but by a pathway involving DAG lipase/MAG lipase action. Studies into the effect of RHC 80267 on LHRH-induced [^{3}H]-AA from α T3-1 cells will, therefore, be of interest. Clearly, further investigations are required to clarify the intracellular mechanisms involved in LHRH-induced [^{3}H]-AA release from α T3-1 cells.

Neither cycloheximide (Figure 4.9) nor quinacrine (Figure 4.5) inhibited PDBu-induced GH release indicating that neither protein synthesis nor PLA_2 activation is required for the mechanism of PKC activation-induced GH release. Instead, PDBu-induced GH release is suggested to occur by a route which is dependent upon 'L'-type voltage-sensitive Ca^{2+} channel activation (Johnson *et al,* 1991). Thus, the PKCs that induce LH and GH release have distinct cellular targets

which may, in part, account for the difference in the time course of PDBu release for each of these hormones.

In conclusion, these experiments show that the induction of LHRH priming requires PLA_2 activation. The form of PLA_2 which is controlled by the LHRH receptor may be related to the cytosolic PLA_2s which associate with the cell membrane, in response to a stimulus, in a Ca^{2+}-dependent manner (Gronich *et al,* 1988, 1990; Channon and Leslie, 1990; Clark *et al,* 1991a; Leslie, 1991). However, changes in Ca^{2+} levels alone are not sufficient for full activation of the gonadotroph PLA_2 since ionomycin-induced LH release was unaltered by PLA_2 inhibitors (Table 4.2). In addition, the action of an H7-resistant PKC-like kinase also appears to be involved in LHRH modulation of PLA_2 activity. Arachidonic acid may induce priming by a mechanism which is independent on the metabolism of the fatty acid. The exact mechanism by which AA enhances gonadotroph responsiveness is unclear, however AA can enhance inositol phospholipid breakdown (Irvine *et al,* 1979), which may account for the facilitated stimulus-secretion coupling which is associated with LHRH priming (Mitchell *et al,* 1988), and can induce Ca^{2+} release from non-mitochondrial stores (Wolf *et al,* 1986; Beaumier *et al,* 1987) which may potentially enhance Ca^{2+}-dependent processes such as gonadotrophin release. Arachidonic acid can synergise with DAG to activate certain PKC isoforms (Shearman *et al,* 1991; Shinomura *et al,* 1991; Chen and Murakami, 1992), possibly causing long-term activation of the 'priming' kinase. However, a sustained action of AA is unlikely to occur during the expression of LHRH priming since PKC inhibitors had no effect on responses to LHRH in pre-primed tissue (Chapter 3). The relevance of these actions of AA in bringing about the priming response is unclear and obviously requires further investigation.

FIGURE 4.1

The effect of quinacrine on LHRH-induced LH (a) and FSH (b) release from pro-oestrous rat anterior pituitary tissue

Tissue was incubated for 3 consecutive hours. In the basal h, tissue was incubated in either medium only (open bars) or with quinacrine (50 μM) (hatched bars) then, in addition, with LHRH (1 nM) during the following 1st and 2nd h incubations. Quinacrine had no significant effect on either basal or 1st h gonadotrophin release, but significantly inhibited 2nd h, LHRH-primed LH and FSH release (*$p < 0.05$). Data are means ± SEM for 4 - 6 determinations.

Figure 4.1

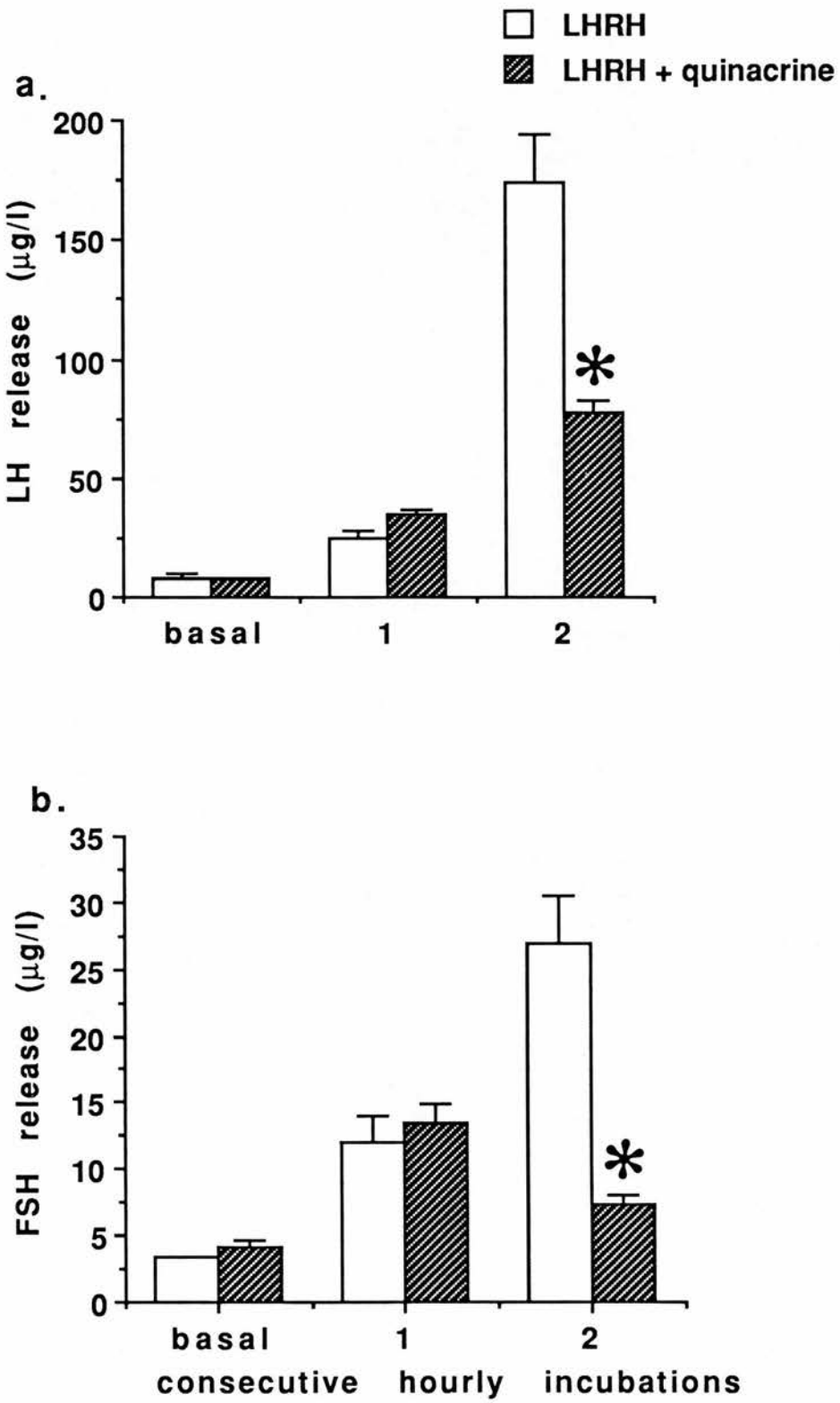

LHRH
LHRH + quinacrine
a.
LH release (µg/l)
200
150
100
50
0
basal
1
2
*
b.
FSH release (µg/l)
35
30
25
20
15
10
5
0
basal
consecutive
1
hourly
2
incubations
*

FIGURE 4.2

Concentration-response curves for the effect of (a) quinacrine and (b) ρ-bromophenacyl bromide on initial LHRH-induced LH release and LHRH priming in pro-oestrous rat anterior pituitary tissue

Tissue was incubated for an initial, basal h (●) in medium only or with increasing concentrations of either quinacrine (10 - 100 μM) or ρ–bromophenacyl bromide (BrPheBr) (10 - 100 μM) followed by 2 further consecutive hourly incubations (1st h (■) and 2nd h (▲)) with, in addition, LHRH (1 nM). Both quinacrine and BrPheBr dose-dependently inhibited 2nd h primed release of LH induced by LHRH, without having any significant inhibitory effect on 1st h LHRH-induced LH release (*$p < 0.05$). Basal h LH release, however, was significantly increased in the presence of BrPheBr at concentrations of 50 - 100 μM. Data are means ± SEM for 4 - 6 determinations.

Figure 4.2

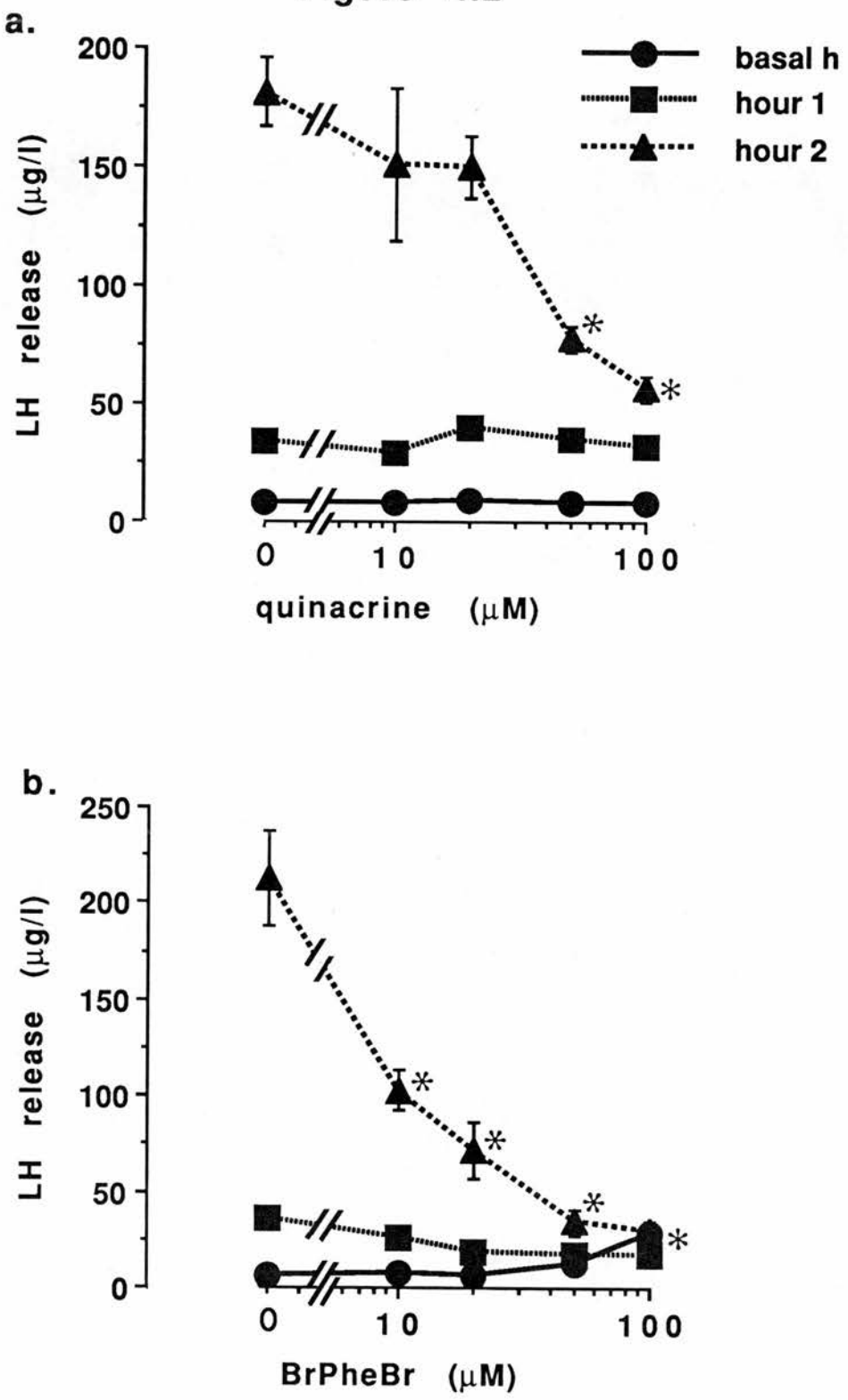

a.
200
150
100
50
0
LH release (µg/l)
basal h
hour 1
hour 2
*
*
0
10
100
quinacrine (µM)
b.
250
200
150
100
50
0
LH release (µg/l)
*
*
*
*
0
10
100
BrPheBr (µM)

FIGURE 4.3

The effect of quinacrine on the development of LHRH priming in pro-oestrous rat anterior pituitary tissue

Hemipituitary pieces were incubated for consecutive hours (basal h, 1st h, 2nd h, 3rd h). In part a, tissue was incubated for a basal h with either quinacrine (hatched bars), or with no drug (open bars), followed by a 1st h with, in addition, LHRH (1 nM). The tissue was then washed in MEM and all treatments were incubated with LHRH only during the final 2nd h. Second h LHRH-induced LH release was significantly inhibited ($*p < 0.05$) in the quinacrine treated tissue. In part b, tissue was incubated for a basal h in medium only followed by a 1st h with 1 nM LHRH (open bars), during which LHRH priming takes place. Throughout the 2nd and 3rd h, tissue was then incubated with LHRH only (open bars) or with LHRH and quinacrine (50 μM) (hatched bars). Quinacrine had no significant inhibitory effect on LHRH-induced LH release from pre-primed tissue. Data are means ± SEM or 4 - 6 determinations.

Figure 4.3

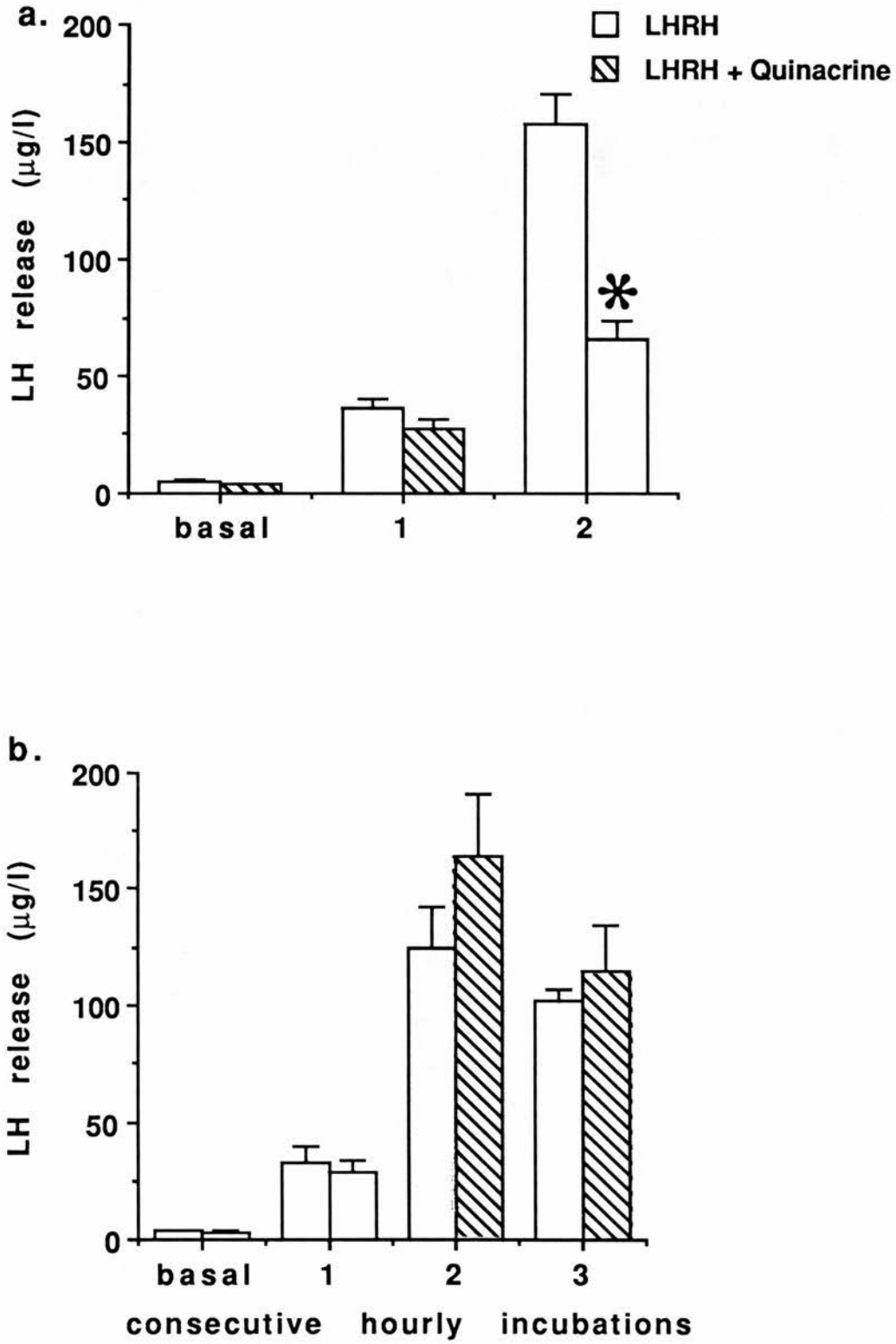

a.
LHRH
LHRH + Quinacrine
LH release (µg/l)
200
150
100
50
0
*
basal
1
2
b.
LH release (µg/l)
200
150
100
50
0
basal
1
2
3
consecutive hourly incubations

FIGURE 4.4

The effect of (a) arachidonic acid and (b) arachidic acid on LH release from pro-oestrous rat anterior pituitary pieces

Hemipituitary pieces were incubated for a basal h in medium only followed by 3 consecutive hourly incubations either in medium only (baseline) or with (a) arachidonic acid (300 μM) or (b) arachidic acid (300 μM). Luteinizing hormone release was significantly increased above baseline during the 2nd and 3rd h of both arachidonic acid and arachidic acid incubation (*$p < 0.05$). Values are means ± SEM for 4 or 5 determinations.

Figure 4.4

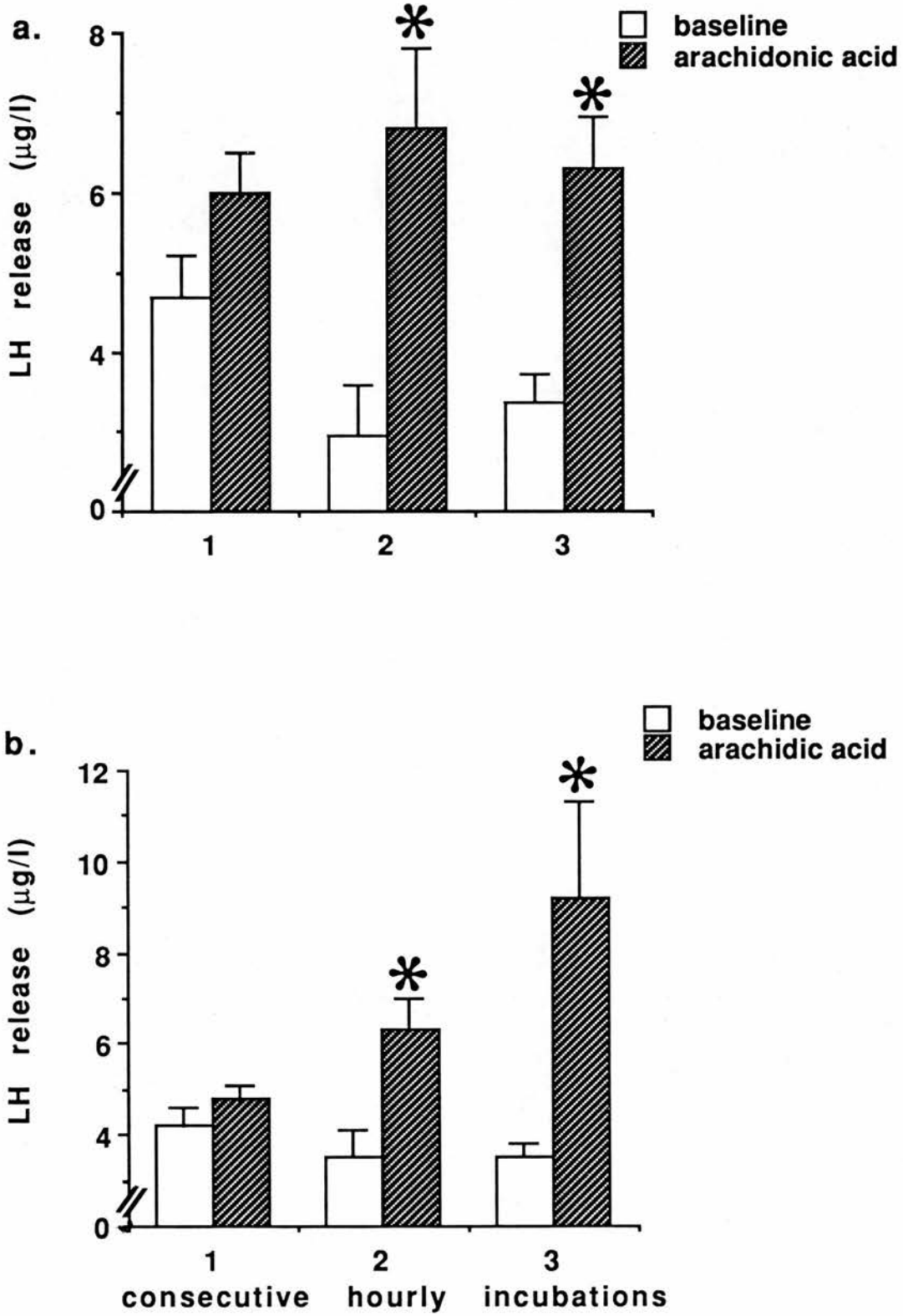

a.
LH release (µg/l)
8
6
4
0
1
2
3
baseline
arachidonic acid
*
*
b.
LH release (µg/l)
12
10
8
6
4
0
1
consecutive
2
hourly
3
incubations
baseline
arachidic acid
*
*

FIGURE 4.5

The effect of quinacrine on phorbol 12,13-dibutyrate (PDBu)-induced LH and GH release from pro-oestrous rat hemipituitaries

Hemipituitaries were incubated for a basal h in either medium only or with various concentrations of quinacrine (2 - 100 μM). Over the following consecutive hourly incubations, the medium contained, in addition, PDBu (300 nM). The data represents LH release measured during the 3rd h of PDBu incubation and GH measured throughout the 1st h of phorbol incubation. Quinacrine dose-dependently inhibited PDBu-induced LH but not GH release ($*p < 0.05$). Each point on the graph represents the mean ± SEM for 4 - 8 determinations.

Figure 4.5

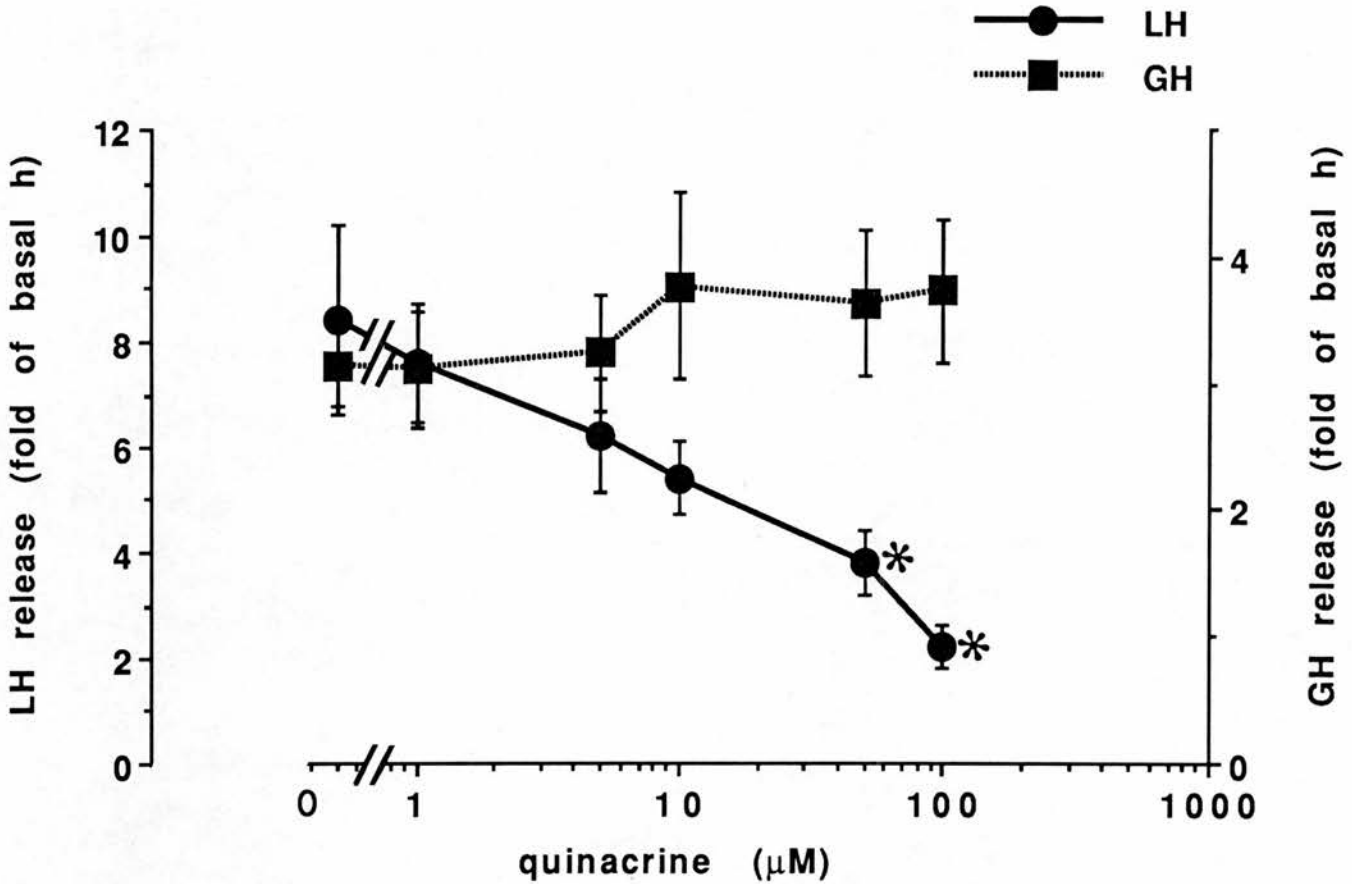

LH
GH
LH release (fold of basal h)
GH release (fold of basal h)
12
10
8
6
4
2
0
4
2
0
0
1
10
100
1000
quinacrine (μM)
*
*

FIGURE 4.6

Concentration response curve for the effect of phorbol 12,13-dibutyrate (PDBu) on [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pre-labelled pro-oestrous rat anterior pituitary tissue

Pairs of pro-oestrous rat anterior pituitary quarters were pre-labelled with [^{3}H]-AA and extensively washed. Tissue was then incubated in medium containing either no drug or various concentrations of PDBu (30 - 1000 nM) for 15 min. Medium [^{3}H]-AA levels were determined. The statistical significance of the effect of PDBu on [^{3}H]-AA release was determined ($*p < 0.05$). Data are means ± SEM for 6 - 8 determinations.

Figure 4.6

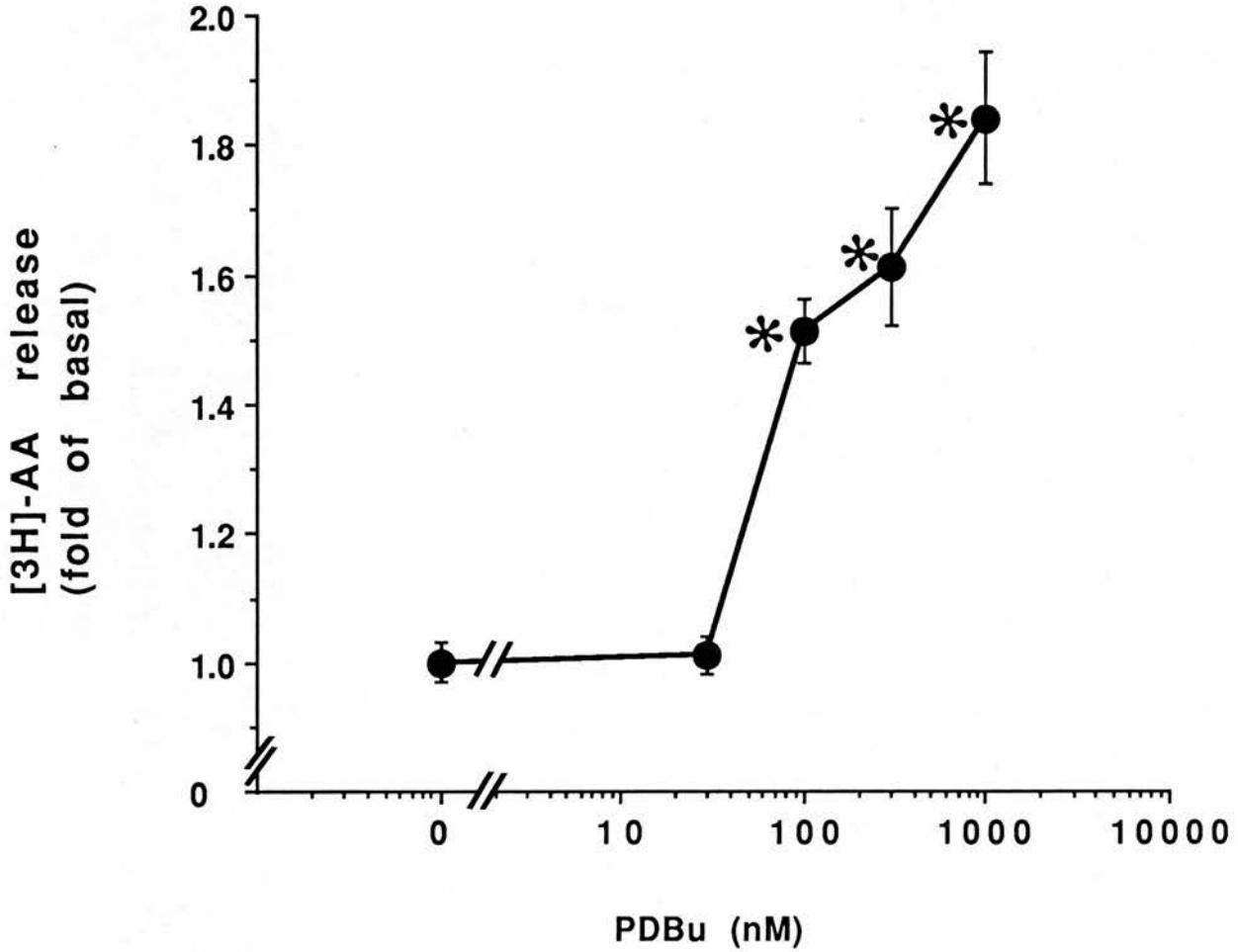

[3H]-AA release
(fold of basal)
2.0
1.8
1.6
1.4
1.2
1.0
0
0
10
100
1000
10000
PDBu (nM)
*
*
*

FIGURE 4.7

Concentration-response curves of the effect of (a) staurosporine, (b) Ro 31-8220 and (c) H7 on phorbol 12,13-dibutyrate (PDBu)-induced [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pro-oestrous rat anterior pituitary tissue

Pairs of pre-labelled anterior pituitary quarters were pre-incubated for 15 minutes with medium only or the appropriate concentration of PKC inhibitor (staurosporine (30 - 1000 nM), Ro 31-8220 (3 -30 μM), H7 (3 - 100 μM)). The medium was discarded and replaced with fresh medium containing either no drug (basal) or PDBu (300 nM) only or PDBu and various concentrations of the appropriate inhibitor. After 15 minutes, [^{3}H]-AA release was determined. Both staurosporine and Ro 31-8220 fully reversed the effect of PDBu. However, PDBu-induced [^{3}H]-AA release apparently displayed 2 components of inhibition by H7. A highly sensitive component of [^{3}H]-AA release was inhibited by H7 at concentrations of < 3 μM, but H7 concentrations from 3 - 30 μM caused little further inhibition and concentrations as high as 100 μM were unable to fully reverse the effects of PDBu. Each point on the graphs represents the mean ± SEM for 4 - 8 determinations.

Figure 4.7

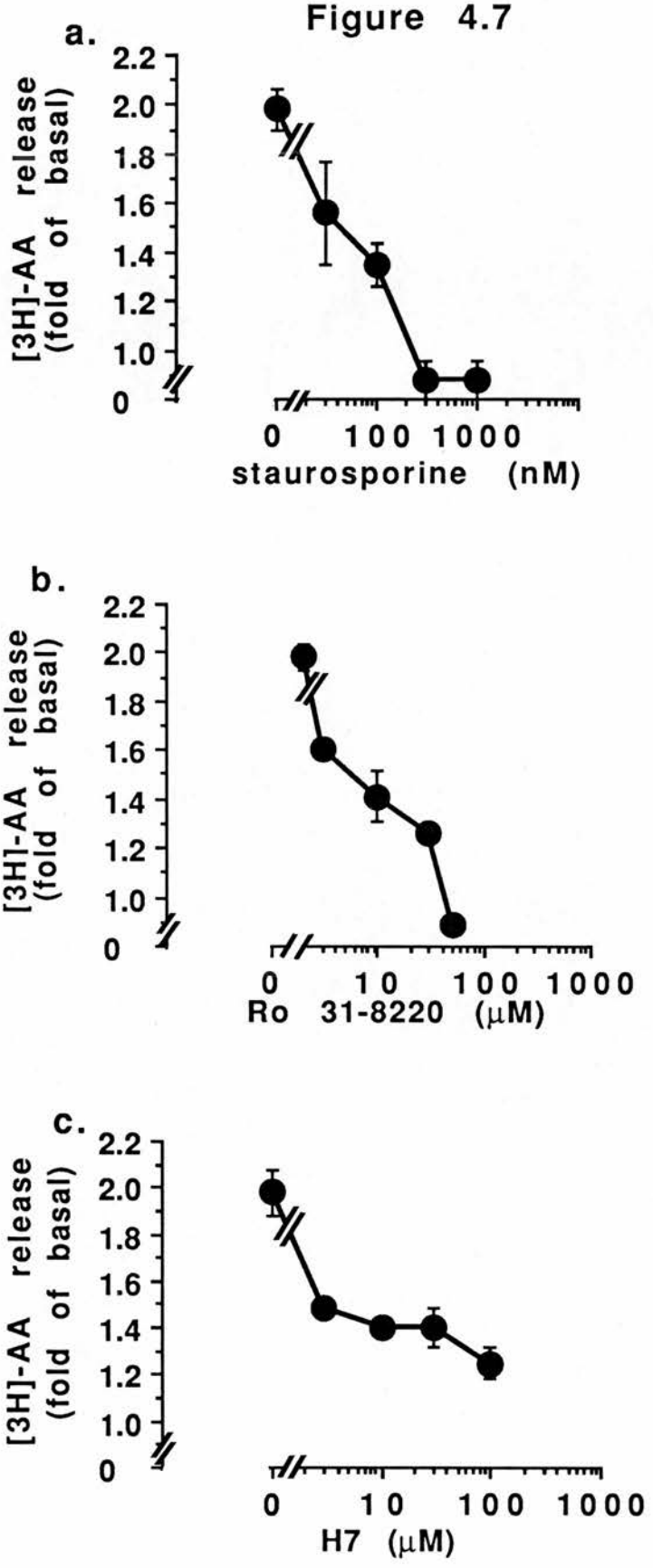

a.
[3H]-AA release (fold of basal)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0 100 1000
staurosporine (nM)
b.
[3H]-AA release (fold of basal)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0 10 100 1000
Ro 31-8220 (μM)
c.
[3H]-AA release (fold of basal)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0 10 100 1000
H7 (μM)

FIGURE 4.8

The effect of cycloheximide on phorbol 12,13-dibutyrate (PDBu)-induced (a) LH and (b) GH release from pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated for a basal h in either medium only (open bars) or with cycloheximide (50 μM) (hatched bars). Over a further 3 consecutive hourly incubations, the medium contained, in addition, PDBu (300 nM). Cycloheximide (CHX) inhibited 2nd and 3rd h PDBu-induced LH but not GH release (*$p < 0.05$). Each point on the graph represents the mean ± SEM for 6 determinations. The SEM values for LH release measured in the CHX-treated samples were within the range of 0.2 - 0.4 ng/ml and were too small to be distinguished by the Y-axis range used here.

Figure 4.8

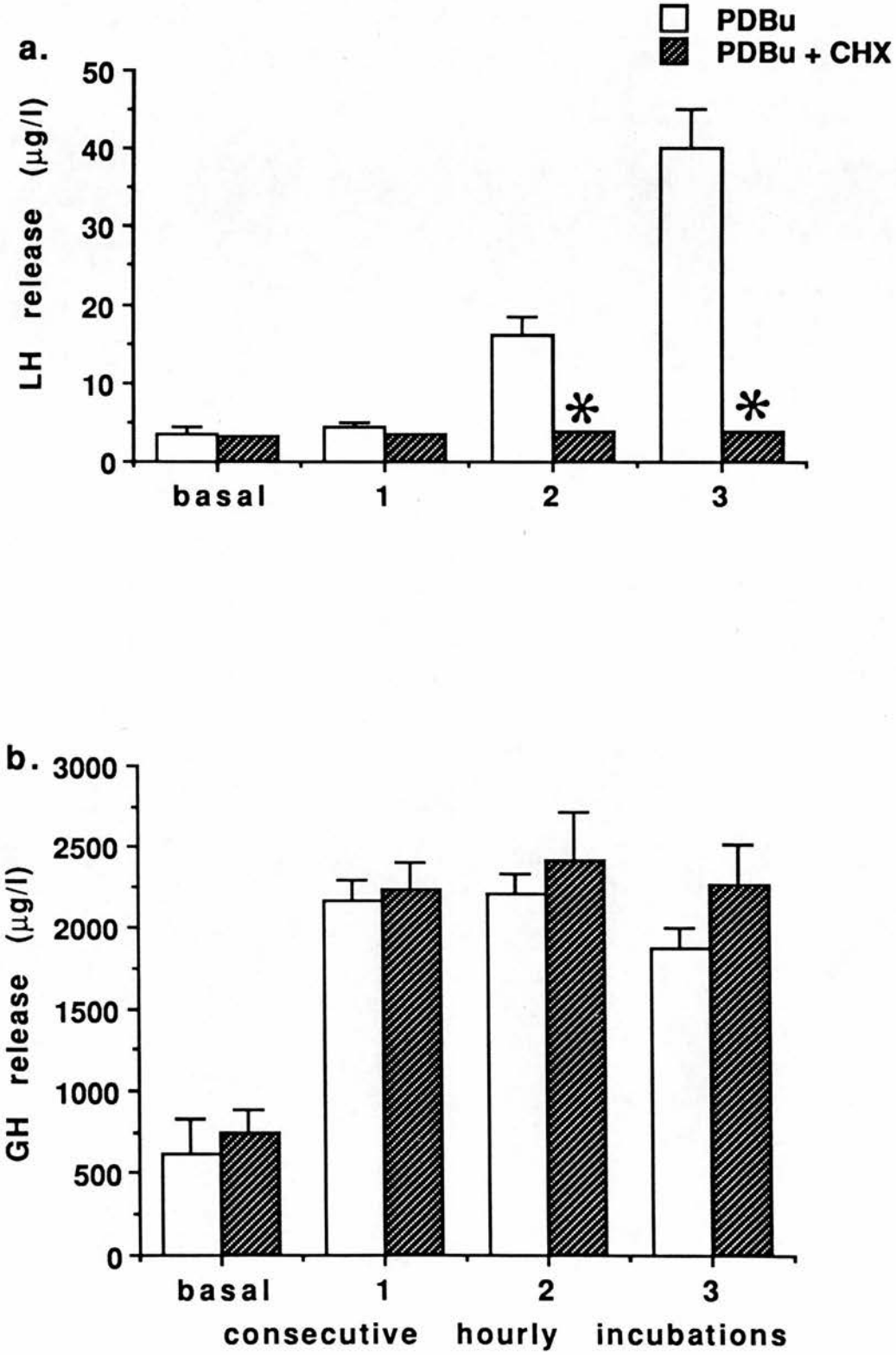

a.
PDBu
PDBu + CHX
LH release (μg/l)
50
40
30
20
10
0
basal
1
2
3
*
*
b.
GH release (μg/l)
3000
2500
2000
1500
1000
500
0
basal
1
2
3
consecutive
hourly
incubations

FIGURE 4.9

The effect of cycloheximide on melittin-induced LH release from pro-oestrous rat hemipituitaries

Hemipituitaries were incubated for a basal h in medium containing either with no drug (open bars) or cycloheximide (CHX, 50 μM) (hatched bars). In the following consecutive hourly incubations, the medium contained, in addition, melittin (3 μM). Melittin-induced LH release was clearly unaffected by the presence of cycloheximide. Data are the means ± SEM for 4 determinations.

Figure 4.9

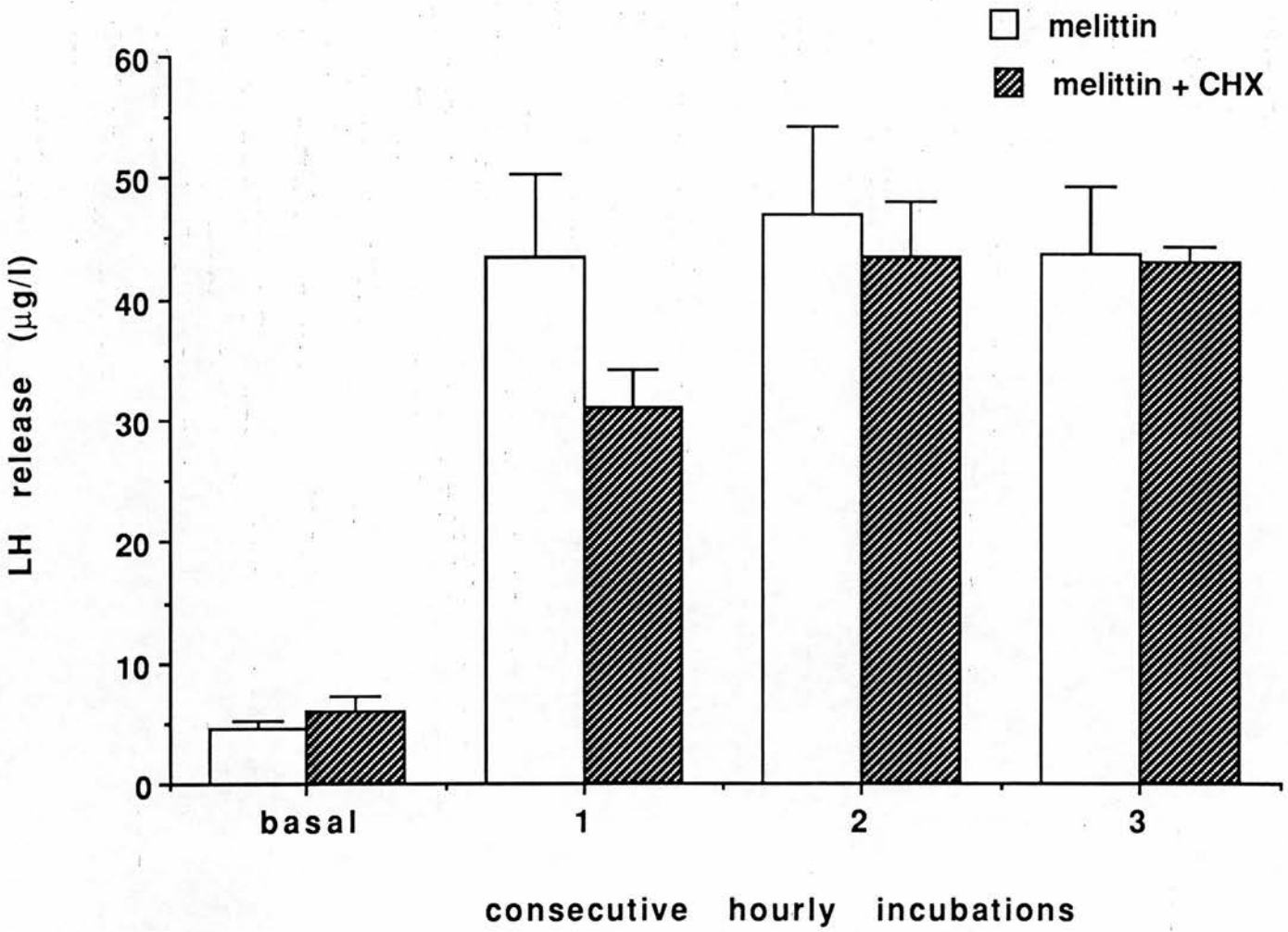

melittin
melittin + CHX
LH release (µg/l)
60
50
40
30
20
10
0
basal
1
2
3
consecutive hourly incubations

FIGURE 4.10

Concentration response curve for the effect of indomethacin on LHRH-induced LH release from pro-oestrous rat hemipituitaries

Hemipituitary pieces were incubated for an initial, basal h (●) in medium only or with increasing concentrations of indomethacin (0.6 - 300 μM) followed by 2 further consecutive hourly incubations (1st h (■) and 2nd h (▲)) with, in addition, LHRH (1 nM). Indomethacin had no inhibitory effect on either basal h or 1st h LHRH-induced LH release over the concentration range used. Second h LHRH-induced LH release was not affected by indomethacin, except at the highest concentration used (300 μM) (*$p < 0.05$). Data are means ± SEM for 4 - 6 determinations.

Figure 4.10

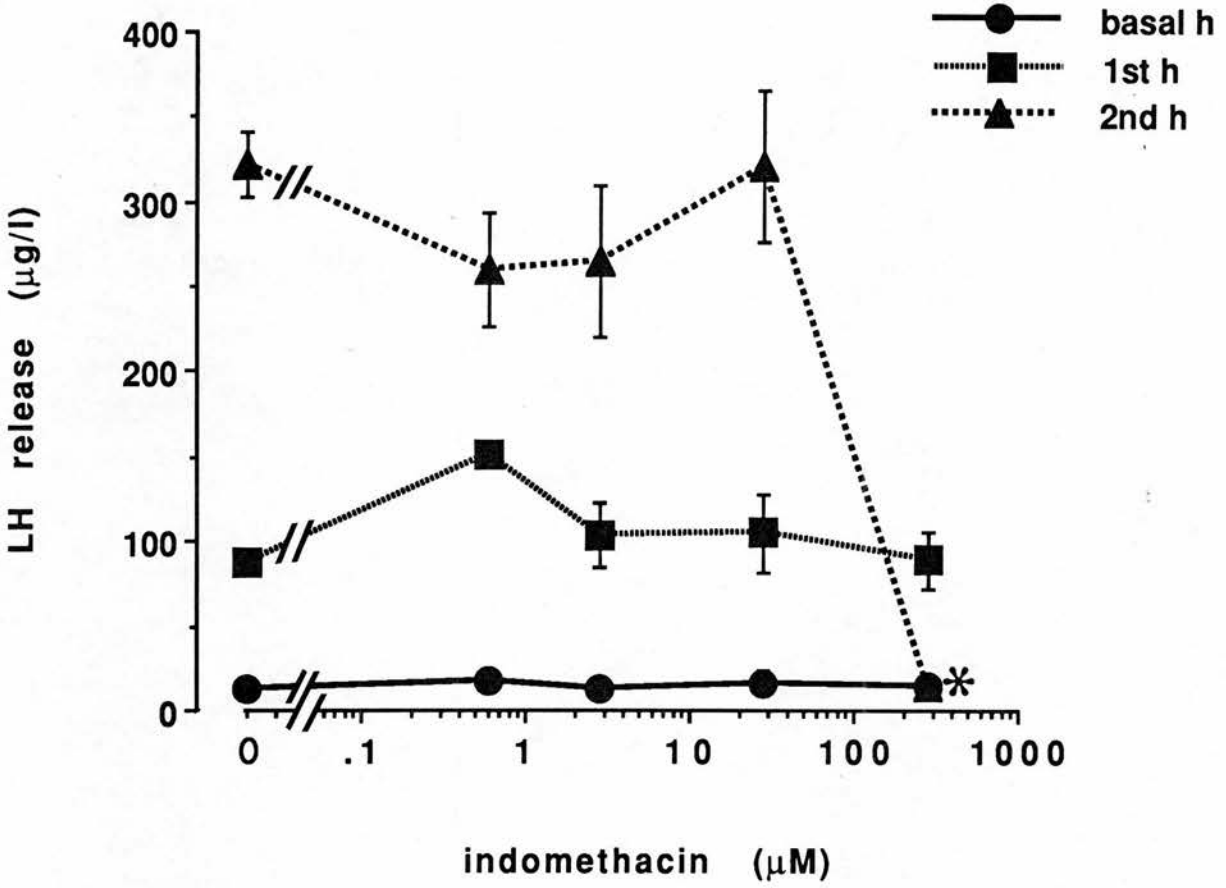

basal h
1st h
2nd h
400
300
200
100
0
LH release (µg/l)
0
.1
1
10
100
1000
*
indomethacin (µM)

TABLE 4.1

The effect of PLA_2 inhibitors on initial LHRH-induced LH release and LHRH priming in pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated *in vitro* for 3 h. In the basal h there was medium alone or a PLA_2 inhibitor (ONO-RS-082 (10 μM), Ro 31-4493 (100 μM), Ro 32-4639 (100 μM), 7, 7-dimethyleicosadienoic acid (DEDA, 30 μM) or aristolochic acid (50 μM)). In the 1st and 2nd h there was, in addition, LHRH (1 nM). The statistical significance of the inhibitory actions of the PLA_2 inhibitors tested was determined (*$p < 0.05$). Values given are the mean ± S.E.M. with the number of determinations in parentheses.

	LH release (μg/l)		
	basal h	**1st h**	**2nd h**
LHRH	6.9 ± 7.0 (5)	24.2 ± 3.9 (5)	90.4 ± 8.8 (5)
LHRH + ONO-RS-082	5.1 ± 0.9 (4)	16.1 ± 2.4 (4)	*20.1 ± 1.6 (4)
LHRH + aristolochic acid	2.9 ± 0.6 (4)	23.4 ± 2.9 (4)	*54.9 ± 9.7 (4)
LHRH + DEDA	5.8 ± 0.5 (4)	21.9 ± 2.3 (4)	88.1 ± 12.2 (4)
LHRH + Ro 31-4493	12.2 ± 1.6 (4)	29.2 ± 2.7 (4)	88.1 ± 7.8 (4)
LHRH + Ro 31-4639	10.6 ± 1.9 (4)	25.6 ± 1.8 (4)	96.2 ± 11.6(4)

TABLE 4.2

The effect of quinacrine on ionomycin-induced LH release from primed or unprimed pro-oestrous rat anterior pituitary tissue

Hemipituitary pieces were incubated for consecutive hours, initially in the presence of medium only or with quinacrine (50 μM) (basal h). In addition, the 1st h incubation had ionomycin (30 μM, I_1) or LHRH (1 nM, L_1), followed by a 2nd h incubation with ionomycin (I_2). Quinacrine had no significant inhibitory effect on ionomycin-induced LH release during any hour of incubation. However, ionomycin-induced LH release from tissue which had been pre-primed by a 1st h incubation with LHRH was significantly inhibited (*$p < 0.05$). All values are means ± SEM and the number of determinations are shown in parentheses.

	LH release (μg/l)		
	basal h	**1st h**	**2nd h**
I_1 - I_2	4.5 ± 1.0 (5)	26.7 ± 2.0 (5)	26.8 ± 2.8 (5)
I_1 - I_2 + quinacrine	7.9 ± 1.3 (4)	27.5 ± 3.1 (4)	25.7 ± 3.0 (4)
L_1 - I_2	7.6 ± 0.4 (5)	28.1 ± 9.1 (5)	75.1 ± 1.0 (5)
L_1 - I_2 + quinacrine	7.5 ± 1.4 (4)	31.1 ± 7.1 (4)	*30.8 ± 1.4 (4)

TABLE 4.3

The effect of PKC inhibitors on arachidonic acid (AA)-induced LH release from pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated *in vitro* for consecutive hours. In the basal h, tissue was incubated in medium only, or with staurosporine (300 nM) or H7 (30 μM)). In the following hours (1st h, 2nd h, 3rd h), the incubation medium contained, in addition, AA (300 μM). The data shown here represent LH release measured during the 3rd h of incubation with AA, during which, LH release was increased significantly with respect to baseline (*$p \leq 0.05$, Student's t-test). Neither H7 nor staurosporine had any significant inhibitory effects on AA-induced LH release (Student's t-test). Values shown are for means ± SEM and the number of determinations are shown in parentheses.

	LH release (μg/l)
baseline	3.4 ± 0.4 (4)
AA	*6.3 ± 1.3 (4)
AA + staurosporine	5.8 ± 0.6 (6)
AA + H7	5.1 ± 0.7 (6)

TABLE 4.4

The effect of PLA_2 inhibitors on phorbol 12,13-dibutyrate (PDBu)-induced LH and GH release from pro-oestrous rat anterior pituitary tissue

Hemipituitaries were incubated for a basal h either in with no drug or with a PLA_2 inhibitor (ρ-bromophenacyl bromide (BrPheBr, 50 μM)) or aristolochic acid (100 μM). In the following hours (1st h, 2nd h, 3rd h), the incubation medium contained, in addition, PDBu (300 nM) or no drug (baseline). The data shown here represent LH release measured during the 3rd h of incubation and GH release measured during the 1st h of incubation with PDBu. In the presence of PDBu alone, both LH and GH release were increased to levels that were significantly greater than baseline ($^{\dagger}p < 0.05$). The statistical significance of the effects of PLA_2 inhibitors on PDBu-induced hormone release is shown by * ($p \leq 0.05$). Values shown are the means ± SEM and the number of determinations are shown in parentheses.

	LH release (μg/l)	**GH release (μg/l)**
baseline	4.9 ± 0.9 (4)	259 ± 14 (4)
PDBu	†32.3 ± 2.7 (6)	†1964 ± 109 (6)
PDBu + BrPheBr	*15.8 ± 2.1 (4)	2072 ± 369 (4)
PDBu + aristolochic acid	*14.2 ± 1.5 (4)	2239 ± 208 (4)

TABLE 4.5

The effect of quinacrine and RHC 80267 on LHRH-induced [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pre-labelled pro-oestrous rat anterior pituitary pieces

Pairs of pre-labelled anterior pituitary quarters were pre-incubated for 15 minutes in medium only, or with quinacrine (50 μM) or RHC 80267 (80 μM). The medium was discarded and replaced with fresh medium containing either no drug (basal) or LHRH (1 nM) only or LHRH together with either quinacrine or RHC 80267. After a second 15 min incubation, the medium was removed and the [^{3}H]-AA was extracted. [^{3}H]-Arachidonic acid release was increased in the presence of LHRH to levels that were significantly greater than baseline ($^{\dagger}p < 0.01$). The statistical significance of the inhibitory effects of quinacrine and RHC 80267 on LHRH-induced [^{3}H]-AA release was determined ($^*p < 0.05$). Values shown are the means ± SEM and the number of determinations are shown in parentheses.

	[^{3}H]-AA release (% of total label incorporated)
baseline	0.85 ± 0.06 (8)
LHRH	†2.17 ± 0.16 (8)
LHRH + quinacrine	*0.92 ± 0.07 (6)
LHRH + RHC 80267	2.14 ± 0.31 (6)

TABLE 4.6

The effect of PKC inhibitors on LHRH-induced [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pre-labelled pro-oestrous rat anterior pituitary pieces

Pairs of [^{3}H]-AA labelled anterior pituitary quarters were pre-incubated for 15 minutes with either no drug, or H7 (30 μM) or staurosporine (300 nM). The medium was replaced and fresh medium contained either no drug (basal), LHRH (1 nM), LHRH and H7, or LHRH and staurosporine. [^{3}H]-Arachidonic acid release was increased in the presence of LHRH to levels that were significantly greater than baseline ($^\dagger p < 0.05$). Staurosporine, but not H7, significantly inhibited LHRH-induced [^{3}H]-AA release ($^* p < 0.05$). Values shown are the means ± SEM and the number of determinations are shown in parentheses.

	[^{3}H]-AA release (% of total label incorporated)
baseline	1.03 ± 0.09 (10)
LHRH	†2.33 ± 0.30 (10)
LHRH + staurosporine	*0.95 ± 0.04 (10)
LHRH + H7	1.79 ± 0.07 (8)

TABLE 4.7

The effect of cycloheximide on LHRH- and phorbol 12,13-dibutyrate (PDBu)-induced [^{3}H]-arachidonic acid ([^{3}H]-AA) release from pro-oestrous rat anterior pituitary pieces

Pairs of pituitary quarters, pre-labelled with [^{3}H]-AA, were pre-incubated for 15 min in medium containing either no drug or cycloheximide (50 μM). The medium was discarded and replacement medium contained, in addition, either no drug (baseline) or LHRH (1 nM) or PDBu (300 nM). After a 15 minute incubation, the medium was removed and [^{3}H]-AA release was determined. [^{3}H]-Arachidonic acid release was increased in the presence of either LHRH or PDBu alone to levels that were significantly greater than baseline ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$). Both LHRH- and PDBu-induced [^{3}H]-AA release were significantly inhibited by cycloheximide ($^*p < 0.05$). Values are means ± SEM for the number of determinations shown in the parentheses.

	[^{3}H]-AA release (% of total label incorporated)
baseline	1.04 ± 0.07 (10)
LHRH	††2.46 ± 0.07 (5)
LHRH + cycloheximide	*1.04 ± 0.09 (6)
PDBu	†1.65 ± 0.10 (8)
PDBu + cycloheximide	*1.16 ± 0.09 (8)

TABLE 4.8

The effect of arachidonic acid and arachidic acid pre-treatment on secretory responses to LHRH measured in pro-oestrous rat anterior pituitary tissue

Hemipituitaries were pre-incubated for a basal h with either arachidonic acid (300 μM), arachidic acid (300 μM) or with no drug (no drug pre-incubation). Following the basal h incubation, all treatments were incubated over 2 consecutive hours (L_1, L_2) with LHRH only (1 nM). Control treatments were pre-incubated with either arachidonic acid or arachidic acid or no drug followed by 2 consecutive hourly incubations in medium only. Luteinizing hormone release during the control incubations was subtracted from corresponding release measured during LHRH treatment. Pre-treatment with arachidonic acid, but not arachidic acid, significantly enhanced net LHRH-induced LH release measured during both 1st and 2nd h incubation periods ($p \leq 0.05$). Values shown are means ± SEM and the number of determinations are shown in parentheses.

	net LHRH-induced LH release (μg/l)	
	L_1	**L_2**
no drug pre-incubation	18.9 ± 2.7 (4)	60.6 ± 8.3 (4)
arachidonic acid pre-incubation	*48.5 ± 6.3 (6)	*115.0 ±11.3 (6)
arachidic acid pre-incubation	23.9 ± 4.2 (4)	60.1 ± 9.1 (4)

TABLE 4.9

The effect of inhibitors of arachidonic acid (AA) metabolism on secretory responses to LHRH measured in pro-oestrous rat anterior pituitary

Hemipituitaries were incubated *in vitro* for consecutive hours. In the basal h, tissue was incubated in medium only, or in the presence of ETYA (10 μM or 30 μM), or NDGA (10 μM or 30 μM). In the following hours (1st h, 2nd h), the incubation medium contained, in addition, LHRH (1 nM). The statistical significance of the effects of each inhibitor was determined (*$p < 0.05$). Values shown are for means ± SEM and the number of determinations are shown in parentheses.

	LH release (μg/l)		
	basal h	**1st h**	**2nd h**
LHRH	4.9 ± 1.4 (5)	33.9 ± 6.6 (5)	123.3 ± 7.4 (5)
LHRH + ETYA (10 μM)	3.6 ± 0.1 (4)	53.2 ± 14.9 (4)	171.1 ± 33.9 (4)
LHRH + ETYA (30 μM)	8.5 ± 1.2 (4)	36.3 ± 6.0 (4)	*12.7 ± 4.2 (4)
LHRH + NGDA (10 μM)	6.0 ± 3.1 (4)	32.0 ± 2.6 (4)	136.8 ± 5.9 (4)
LHRH + NDGA (30 μM)	3.7 ± 0.4 (4)	*6.3 ± 1.2 (4)	*6.32 ± 3.16 (4)

TABLE 4.10

The effect of LHRH and phorbol 12,13-dibutyrate (PDBu) on [^{3}H]-arachidonic acid (AA) release from α T3-1 cells

α T3-1 cells which had been pre-labelled with [^{3}H]-AA and extensively washed,were pre-incubated for 15 minutes with either no drug, staurosporine (300 nM), or H7 (30 μM). The medium was replaced and the cells were incubated for a further 15 minutes with, in addition either no drug (basal), LHRH (100 nM) or PDBu (300 nM) and medium [^{3}H]-AA levels were determined. [^{3}H]-Arachidonic acid release was significantly increased in the presence of LHRH, but not PDBu when compared to baseline (*$p < 0.05$). Neither staurosporine nor H7 had any significant inhibitory effect on LHRH-induced [^{3}H]-AA release. Data are shown as means ± SEM and the number of determinations are shown in parentheses.

	[^{3}H]-AA release (% label incorporated)
baseline	0.80 ± 0.15 (8)
LHRH	*5.12 ± 0.35 (6)
LHRH + staurosporine	5.76 ± 0.13 (6)
LHRH + H7	4.06 ± 0.35 (6)
PDBu	0.73 ± 0.22 (8)

CHAPTER 5

THE EFFECT OF GONADAL STEROIDS ON PROTEIN KINASE C AND PHOSPHOLIPASE A_2-MEDIATED RESPONSES IN RAT ANTERIOR PITUITARY CELLS

5.1 INTRODUCTION

The responsiveness of gonadotrophs to secretagogues, such as LHRH (Arimura and Schally, 1974; Aiyer and Fink, 1974; Drouin et al, 1976; Kamel et al, 1987a; Turgeon and Waring, 1981), depolarising concentrations of potassium (Liu and Jackson, 1988) and activators of PKC (Fahmy et al, 1989; Liu and Jackson, 1988; Audy et al, 1990; Drouva et al, 1990) can be markedly altered by the prior presence of gonadal steroids, suggesting that steroids modulate several aspects of stimulus-secretion coupling in these cells. Since the magnitude of priming is altered during the oestrous cycle (Waring and Turgeon, 1980), it seems that gonadal steroids may have a modulatory influence upon the mechanism of priming. The experiments that are described in Chapters 3 and 4 suggest that the induction of LHRH priming is dependent upon the actions of an H7-resistant PKC-like kinase which modulates the activity of PLA_2. We therefore investigated the possibility that steroid modulation of pituitary responsiveness may involve an effect on the pharmacologically distinct forms of PKC that are involved in mediating LH release and inducing LHRH priming and, as a consequence, on LHRH activation of PLA_2.

5.2 SPECIFIC METHODOLOGY

5.2.1 Hormone Secretion Experiments

Female COB Wistar rats were anaesthetised and decapitated by 11.30 am on the appropriate day of the oestrous cycle. Long-term ovariectomised rats were anaesthetised and decapitated by 11.30 am on the 5th day after implantation with silicone elastomer capsules containing either no steroid or oestrogen (E_2). Anterior pituitary glands were removed, hemisected and hormone release was measured as described in Chapter 2 (section 2.2.3). Hormone release was determined as described in Chapter 2. The medium that was collected at the end of each hourly incubation and was stored at -20°C until it was radioimmunoassayed for LH and GH (Niswender *et*

al, 1968; Daane and Parlow, 1971). The standards used were NIADDK-rat LH-RP2 and NIADDK-rat-GH-RP-2.

5.2.2 Cytosolic protein kinase C activity assay

Anterior pituitary PKC activity was determined as the phosphatidylserine-dependent, histone IIIS kinase activity induced by PKC activators using methods described in Chapter 2, section 2.2.4. Protein kinase C activity was measured either in the absence of activator (i.e. basal activity) or in the presence of 1 μM PDBu or 100 μM DOG. Kinase activity measured in the presence of phosphatidylserine, 5 mM EGTA (i.e. low free Ca^{2+}) and in the absence of PKC activator was taken to represent basal activity and was subtracted from all activator-stimulated levels. Calcium-independent kinase activity was taken to be the activity measured in the presence of 5 mM EGTA and PKC activator. The additional kinase activity measured with samples containing $CaCl_2$ + PKC activator was taken to represent calcium-dependent kinase activity.

5.2.3 Cytosolic [^{3}H]-phorbol 12,13-dibutyrate binding

The number of PKC molecules in E_2-treated and -untreated pituitary cytosol and their affinity for PDBu were assessed using the [^{3}H]-PDBu binding method described in Chapter 2, section 2.2.6.

5.2.4 [^{3}H]-Arachidonic acid release measurements

Long-term ovariectomised female COB Wistar rats were implanted with capsules containing either E_2 or no steroid (Chapter 2, section 2.2.2). On the 5th day after implantation, rats were anaesthetised, decapitated and anterior pituitaries were removed and bisected. Each hemipituitary was cut into 2 equal pieces and tissue was labelled with [^{3}H]-AA. [^{3}H]-Arachidonic acid release from pre-labelled tissue was determined using the methods described in Chapter 2, section 2.2.5.

To examine the effects of steroids on responses measured in primary cultures of anterior pituitary cells, anterior pituitary glands, removed from female rats at random stages of the oestrous cycle, were enzymatically dispersed and the cells cultured for 4 days in steroid-free DMEM, without phenol red (see Appendix I.1 and I.2), as described in Chapter 2, section 2.2.2. Cells were then incubated in DMEM with either no steroid or 1 nM E_2 for 48 h. [^{3}H]-Arachidonic acid release from pre-labelled cells was determined as described in Chapter 2, section 2.2.5.

5.2.5 Drugs

Phorbol 12,13-dibutyrate, DOG, ionomycin and staurosporine were made up as stock solutions in DMF. The maximum concentration of DMF (0.5% v/v) was used in control experiments and had no effect on either hormone or [^{3}H]-AA release. Quinacrine, LHRH, H7, leupeptin and E64 were all made up as stock solutions in distilled H_2O.

All PKC assay components and drugs were dissolved in 20 mM Tris HCl (pH 7.5) + 0.5 mM EGTA except for DOG which was added to each assay tube in 1 µl DMF using a Hamilton microsyringe.

5.2.6 Data analysis

The statistical analysis of the time course data of hormone release obtained from the experiments using tissue from ovariectomised animals, with or without E_2 treatment, was carried out by both 2-way and 1-way analysis of variance, followed by Duncan's New Multiple Range test on significantly different groups. All other statistical analysis were carried out using the Mann-Whitney U-test or Student's t-test

5.3 RESULTS

The effect of PDBu and LHRH on hormone release from rat anterior pituitary tissue removed at different stages of the oestrous cycle

Table 5.1 shows the effect of consecutive hourly incubations of LHRH (1 nM) on LH release from rat hemipituitaries removed at different stages of the oestrous cycle. Basal h LH release did not alter significantly with the stage of the cycle. However, the magnitude of both 1st h and 2nd h LHRH-induced LH release were oestrous cycle day-dependent. Tissue taken on the morning of metoestrus was the least responsive to a 1st h incubation with LHRH, and tissue responsiveness gradually increased over dioestrus, reached a maximum on the morning of pro-oestrus, and decreased by the morning of oestrus. Similarly, 2nd h LHRH-induced LH release, where LHRH priming is expressed, was smallest in tissue removed at metoestrus, increased over dioestrus and was maximal by pro-oestrus. Neither basal h nor 1st h LHRH-induced LH release, measured at any stage of the oestrous cycle, was inhibited by staurosporine. However, at all stages of the cycle, the 2nd h primed release of LH was significantly inhibited by staurosporine to 1st h levels of LHRH-induced LH release. Thus, the LHRH priming response measured at all stages of the oestrous cycle may be dependent upon activation of a protein kinase, in particular, a form of PKC.

Since the magnitude of priming is oestrous cycle-dependent, any of the factors which participate in the induction of the priming response, such as PKC, may be modulated in a cycle-dependent manner. To examine the possibility that PKC action in gonadotrophs may alter over the oestrous cycle, the effect of consecutive hourly incubations with PDBu (300 nM) was examined on LH release, and as a comparison, GH release from tissue removed from female rats at different stages of the cycle (Figure 5.1). The magnitude of PDBu-induced LH release was found to alter with the stage of the oestrous cycle. Tissue removed on the morning of

metoestrus was least responsive, with 3rd h PDBu-induced LH release increasing only to around 2-fold of basal h levels. The magnitude of PDBu-induced LH release increased over dioestrus, and was maximal on pro-oestrus, on which, 3rd h LH release was 7-fold of basal h levels. Tissue removed on the morning of oestrus was slightly less responsive to PDBu than tissue removed at pro-oestrus. Interestingly, PDBu-induced LH release from oestrous tissue displayed a slightly different temporal pattern from that measured on other days of the cycle. That is, PDBu-induced LH release from oestrous rat tissue, but not from tissue taken at any other stage of the cycle, was increased above basal h levels by the 1st h of incubation with PDBu. It is also interesting to note that the relative responsiveness to PDBu throughout the oestrous cycle (with respect to LH release) does not linearly parallel the capacity to prime in response to LHRH. Therefore, although PKC may be an important component in the induction of LHRH priming, other steroid-regulated factors may also play a significant role in this response. In contrast to PDBu-induced LH release, neither the magnitude nor time-course of PDBu-induced GH release altered significantly with the oestrous cycle. Therefore, factors (such as ovarian steroids) which control the reproductive cycle of the rat may modulate the transduction pathways, possibly PKC or subsequent targets, through with phorbol esters can induce LH, but not GH, release.

The effect of E_2 treatment of long-term ovariectomised rats on anterior pituitary responses to LHRH measured *in vitro*

The changes in the magnitude of PDBu-induced LH release and LHRH priming measured over the oestrous cycle correlate with the changes in circulating E_2 levels that are measured in the rat, *in vivo* (Turgeon and Waring, 1981; Evans *et al,* 1983). That is, during metoestrus, when rat plasma E_2 levels are at their lowest, pituitary LH responses are at their smallest. As the circulating E_2 levels increase over dioestrus and reach a peak on pro-oestrus, pituitary responsiveness to PDBu and

LHRH increases accordingly. Therefore, it appears that E_2 can somehow enhance stimulus-secretion coupling in gonadotrophs. To test for a possible action of E_2 on LHRH receptor responses, the effects of *in vivo* E_2 treatment of long-term ovariectomised rats was examined on anterior pituitary responses to LHRH, *in vitro* (Table 5.2). Throughout both hours of LHRH incubation, LH release from ovariectomised rat hemipituitaries was increased above basal h levels. However, the magnitude of 1st and 2nd h LHRH-induced LH release was not significantly different indicating that ovariectomised rat tissue may lack the ability to prime. Following E_2 treatment, both 1st h and 2nd h LHRH-induced LH release were potentiated in comparison to release measured from untreated ovariectomised rat tissue. Furthermore, following E_2 treatment, the ability of the tissue to exhibit a form of priming was apparently restored, with the 2nd h secretory response to LHRH being approximately 2-fold greater than 1st h LHRH-induced LH release. However, it should be noted that this priming ratio of 2nd h/1st h release is still somewhat smaller than the LHRH priming ratio of around 7-fold that was seen on pro-oestrus (Table 5.1).

In the presence of staurosporine (300 nM), neither basal h nor 1st h LHRH-induced LH release was inhibited, irrespective of the steroid exposure of the tissue. However, 2nd h LHRH-induced LH release from both E_2-treated and -untreated tissue appeared to be partially inhibited by staurosporine, although, due to the small sample size of the E_2-treated group, the statistical significance of this effect could not be determined. In contrast, LHRH-induced LH release measured during any hour was unaffected by H7 (30 μM) in tissue removed from either E_2-treated or -untreated ovariectomised animals. These preliminary results further suggest that later secretory responses to LHRH in tissue removed from either E_2-treated or -untreated ovariectomised animals are dependent upon activation of staurosporine-sensitive but H7-resistant kinase, perhaps the H7-resistant PKC activity that was detected in pro-oestrous rat anterior pituitary cytosol (Chapter 3). Furthermore, E_2

treatment may facilitate the expression of LHRH priming during the later phase of hormone release, possibly by modulating the actions of this kinase.

The effect of E_2 treatment of long-term ovariectomised rats on anterior pituitary responses to PKC activators measured *in vitro*

To test the hypothesis that changes in E_2 levels throughout the oestrous cycle may be a cause of the variation in magnitude of PKC responses in rat gonadotrophs, long term ovariectomised rats were implanted with capsules containing either E_2 or no steroid for 5 days, and secretory responses to PKC activators were measured *in vitro*. The baseline release of both hormones was determined in parallel control incubations for 4 consecutive hours and the corresponding values were subtracted form all stimulated values to allow presentation of net stimulus-induced responses. Consecutive hourly incubations with PDBu (300 nM) significantly increased net LH and GH release from anterior pituitary tissue taken from both E_2-treated and -untreated ovariectomised rats (Figures 5.2 and 5.3). The magnitude of PDBu-induced LH release from pituitary tissue removed from E_2-treated rats was very markedly greater than that seen using tissue from untreated ovariectomised animals ($p < 0.05$, $p < 0.01$, Mann-Whitney U-test). For example, in the 3rd h of PDBu incubation, net LH release from E_2-treated and -untreated ovariectomised rats was 265.7 ± 31.6 µg/l ($n = 10$) and 20.7 ± 3.9 µg/l ($n = 10$) respectively (Figure 5.2a). Furthermore, the time course of PDBu-induced LH release altered with E_2 treatment. In tissue from E_2-treated, but not -untreated rats, there was a time-dependent increase in LH release in response to PDBu ($F(3,36) = 37.35$, $p \leq 0.0001$ and $F(3,36) = 1.73$, $p = 0.178$, respectively). Statistically significant increments in LH release from E_2-treated tissue occurred progressively at each hour of PDBu incubation ($p < 0.05$, Duncan's New Multiple Range test). Thus, LH release from untreated tissue reached maximal levels by the 1st h of PDBu incubation, whereas LH output from E_2-treated

tissue increased progressively with each successive PDBu incubation (in a manner similar to that seen with pro-oestrous tissue (see Chapter 3).

The release of GH was increased by PDBu in both E_2-treated and -untreated tissue ($F(3,36) = 9.17$, $p \leq 0.0001$ and $F(3,36) = 5.47$, $p \leq 0.0034$ respectively) with statistically significant increments over basal in the 1st, 2nd and 3rd h of PDBu incubation in each group (Duncan's New Multiple Range test) (Figure 5.3a). However, in contrast to PDBu-induced LH release, the magnitude of PDBu-induced GH release was not significantly different between any hour of incubation in other group or between E_2-treated and -untreated tissue respectively (Mann-Whitney U-test). These data are consistent with E_2 having a facilitatory action on the mechanism by which phorbol ester activators of PKC can induce LH but not GH release.

In a manner similar to PDBu, DOG (200 μM) also induced a significant increase in LH release from pituitary tissue from both untreated and E_2-treated animals (Figure 5.2b). Analysis of raw data failed to show a statistically significant time-dependent increase in overall LH output form either E_2-treated or -untreated tissue in the presence of DOG. However, the corresponding basal LH release in each hour (assessed in parallel control incubations), showed a time-dependent decrease. When these basal values were subtracted to yield net DOG-induced LH release, non-parametric analysis (Mann-Whitney U-test) revealed a significant increment in LH release at each hour of incubation with DOG ($p < 0.05$). There were no significant differences between DOG-induced LH release from E_2-treated or -untreated tissue at any time point. Using pituitary tissue from untreated ovariectomised rats, DOG-induced LH release was similar in magnitude to PDBu-induced LH release at all hours of incubation with PKC activator (Figure 5.2). However, DOG was unable to mimic the facilitatory actions of PDBu on LH release from E_2-treated tissue in that the magnitude of DOG-induced LH release from E_2-treated and -untreated tissue was similar. In contrast to the effects of PDBu, DOG (at concentrations up to 200 μM)

was unable to induce GH release from either E_2-treated or -untreated tissue (Figure 5.3), similar to pro-oestrous rat tissue (see Chapter 3), suggesting that the PKC(s) which mediate GH and LH release differ in their susceptibility to activation by DOG.

The effect of H7 and staurosporine on PKC activator-induced LH and GH release from anterior pituitary tissue removed from ovariectomised rats treated with or without E_2

In a further set of experiments, the effects of staurosporine and H7 were examined on PKC activator-induced hormone release from pituitary tissue from E_2-treated and -untreated ovariectomised rats (Table 5.3). The release of LH induced by PDBu (300 nM) from both E_2-treated and -untreated ovariectomised rat pituitary tissue was significantly inhibited by 300 nM staurosporine ($p < 0.05$). However, in contrast to previous results obtained with pro-oestrous pituitary tissue, where 100 nM PDBu-induced LH release was readily blocked by H7 ($IC_{50} = 1.7 \pm 1.5$ μM, $n = 5$) (Chapter 3), H7, at concentrations up to 30 μM, was unable to significantly inhibit either DOG- or PDBu-induced LH release from anterior pituitary tissue taken from either E_2-treated or -untreated ovariectomised rats (Table 5.3). Phorbol-ester induced GH release was similarly inhibited by staurosporine, but was insensitive to H7 at concentrations up to 30 μM (Table 5.3). Neither staurosporine nor H7 at the concentrations used had any significant effect on basal LH or GH release.

The effect of E_2 treatment on PKC activity and [^{3}H]-PDBu binding site number in cytosol from ovariectomised rat anterior pituitary tissue

The possibility that E_2 treatment may enhance PKC-induced LH release by altering anterior pituitary PKC activity was investigated. Using histone IIIS as a substrate, PDBu (1 μM) was able to elicit approximately equal proportions of Ca^{2+}-dependent and -independent PKC activity in pituitary cytosol from untreated ovariectomised rats (Table 5.4). With E_2 treatment there was an approximate 2-fold increase in both Ca^{2+}-dependent and -independent, PDBu-induced PKC activity. In

both treatment groups, DOG (100 μM) apparently induced relatively less Ca^{2+}-dependent and -independent cytosolic kinase activity than PDBu (Table 5.4). However, direct comparisons of the kinase activity elicited by DOG and PDBu cannot be made unless comprehensive concentration response curves are available for each of these agents. Similar to the results seen using PDBu, DOG- stimulated Ca^{2+}-dependent PKC activity approximately doubled with E_2 treatment. Whilst there was also a slight increase in DOG-stimulated, Ca^{2+}-independent PKC activity with E_2 treatment, this was not statistically significant.

Studies were carried out to examine the actions of H7 and staurosporine on PDBu-stimulated PKC activity in anterior pituitary tissue cytosol (Figure 5.4, Table 5.5). In anterior pituitary cytosol from untreated ovariectomised animals, PDBu (1 μM)-stimulated, Ca^{2+}-dependent PKC activity was much more sensitive to inhibition by H7 than Ca^{2+}-independent activity. In E_2-treated tissue, although PDBu-stimulated activity was greater in magnitude, Ca^{2+}-dependent, PDBu-stimulated kinase activity was also found to be more sensitive to H7 than Ca^{2+}-independent activity. In contrast, the potency of staurosporine at inhibiting both Ca^{2+}-dependent and -independent PKC activity did not differ significantly and was unaltered by E_2 treatment (Table 5.5). Since PDBu-induced LH and GH release from E_2-treated and -untreated tissue are relatively insensitive to inhibition by H7 at concentrations of 30 μM (Table 5.3), the results from the PKC activity assays suggest that those PKC(s) which induce both LH and GH release may not be dependent upon raised intracellular Ca^{2+} for their effective activation by phorbol esters.

Binding studies showed that the number of specific [^{3}H]-PDBu binding sites in anterior pituitary cytosol significantly increased by approximately 2-fold upon E_2 treatment without any significant change in site affinity for PDBu (Table 5.6). It seems likely therefore, that the increase in PDBu-stimulated PKC activity observed after E_2 treatment may occur as a result of an increase in pituitary PKC content, and not as a result of a change in the affinity of existing PKC(s) for PDBu.

The effect of E_2 treatment on ionomycin-induced LH and GH release from ovariectomised rat anterior pituitary tissue *in vitro*

It is possible that the facilitatory actions of E_2 treatment on PKC-induced LH release may involve steroid-mediated enhancement of other steps in the process by which PKC can induce LH secretion, in addition to altering cytosolic PKC activity and content. To examine the possibility that E_2 treatment can enhance LH release by increasing the amount of gonadotrophin available for release, we measured hormone release in response to maximally raised intracellular Ca^{2+} levels using ionomycin, a procedure which may be taken as an indirect measure of releasable pituitary hormone content. Over 3 consecutive hourly incubations, ionomycin (30 μM) significantly increased LH and GH release from anterior pituitary tissue from both E_2-treated and -untreated ovariectomised animals (Figure 5.5). However, in contrast to the effect of PDBu on LH release, ionomycin-induced LH release from either E_2-treated or -untreated tissue was not significantly different at any hour of incubation (Figure 5.5a). This result suggests that E_2 does not enhance PKC-induced LH release by increasing the size of the releasable LH pool. Ionomycin-induced GH release was also found to be unaltered with E_2 treatment (Figure 5.5b), suggesting that E_2 does not alter the readily-releasable pool of GH.

The effect of quinacrine on PDBu-induced LH and GH release from anterior pituitary tissue removed at different stages of the oestrus cycle

The experiments described in Chapter 4 suggest that PKC-dependent modulation of PLA_2 activity is an integral component in the induction of LHRH priming. To test for possible oestrous cycle-dependent changes in PLA_2 involvement in PKC-elicited LH responses, the effect of quinacrine was examined on PDBu-induced LH release, and as a comparison, GH release (Figures 5.6 and 5.7). On metoestrus, when tissue LH responses to PDBu are at their minimum, quinacrine had no significant inhibitory effect on PDBu-induced LH release during any hour of

phorbol incubation (Figure 5.6). Similarly, PDBu-induced LH release from tissue removed at dioestrus was not significantly altered by the presence of quinacrine at any hour of incubation, although the 3rd h of LH release was slightly reduced by the presence of this inhibitor. On pro-oestrus, where hemipituitary LH responses are at their greatest, 3rd h PDBu-induced LH release was significantly reduced in the presence of quinacrine ($p < 0.05$). Second h PDBu-induced LH release from pro-oestrous tissue was also reduced by quinacrine, although not to significant levels. Quinacrine also significantly inhibited 3rd h PDBu-induced LH release from hemipituitaries which had been removed on oestrus. It is of interest that the inhibitory actions of quinacrine on LH release were detected on the more responsive days of the oestrous cycle (i.e. pro-oestrus and oestrus) and that quinacrine reduced LH release on these days to PDBu-stimulated levels measured on the least responsive days (metoestrus and dioestrus), but never to baseline levels. Therefore, enhanced LH responsiveness to PDBu measured over the oestrous cycle may, in part, result from cycle-dependent changes in the involvement of PLA_2 in the mechanism of PKC-induced LH release.

In contrast to the effects of quinacrine on PDBu-induced LH release, PDBu-induced GH release, measured at any hour of incubation, was not significantly altered by the presence of quinacrine on any day of the oestrous cycle. These results further support previous observations made in Chapter 3 which indicate that the kinase(s) that induce GH release do not produce the release of this hormone by a mechanism involving PLA_2 activation.

The effect of E_2 treatment on the involvement of PLA_2 in responses measured in ovariectomised rat anterior pituitary tissue and in 5 day cultures of dispersed anterior pituitary cells from random cycling female rats

Since the involvement of PLA_2 in PDBu-induced LH release was oestrous cycle day-dependent, it is possible that gonadal steroids, and E_2 in particular, can

modulate the ability of PKC activators to elicit PLA_2 activity. To test this hypothesis, we examined the effect of quinacrine on PDBu-induced LH and GH release from hemipituitaries removed from long-term ovariectomised rats which had received treatment with either E_2 or no steroid (Table 5.7). Using untreated ovariectomised rat tissue, 3rd h PDBu (300 nM)-induced LH release was not inhibited in the presence of quinacrine (50 μM). The secretory responsiveness of gonadotrophs to PDBu was much greater in tissue removed from rats which had received prior treatment with E_2, and was significantly inhibited by quinacrine ($p < 0.05$). These results indicate that E_2 can modulate the mechanism by which phorbol esters induce LH release, possibly by having an effect on the extent of PLA_2 involvement in this response. Consistent with this hypothesis, [^{3}H]-AA pre-labelled hemipituitaries from untreated ovariectomised rats did not release significant levels of [^{3}H]-AA in response to 15 minutes exposure to PDBu (Table 5.8). However, PDBu could induce a significant increase in [^{3}H]-AA release from pre-labelled anterior pituitary tissue that had been removed from E_2-treated ovariectomised animals. The magnitude of 1st h PDBu-induced GH release was not altered by prior E_2 treatment of the animal, nor was it blocked by quinacrine.

Oestrogen may, in principle, modulate the involvement of PLA_2 in PDBu-mediated LH responses by having a number of actions. For example, E_2 may modulate the activity of PLA_2 itself and/or the PKC(s) which control PLA_2 activity and/or alter the protein synthesis-dependent mechanism by which PKC modulates PLA_2 activity. To gain an insight into which of these possible effects may contribute to the actions of E_2 on PLA_2 involvement, a preliminary study was carried out. In the presence of ionomycin (30 μM), [^{3}H]-AA release from pre-labelled dispersed anterior pituitary cell cultures was increased by approximately 40 - 50% above basal levels in both 48 h E_2-treated and -untreated cells (Table 5.9). However, in the presence of PDBu (300 nM), [^{3}H]-AA release from steroid-untreated cells was not different from baseline levels of release. In contrast, in E_2-treated cultures, PDBu induced an almost

2-fold increase in [^{3}H]-AA release. These results suggest that E_2 does not directly modulate PLA_2 activity, since ionomycin-induced activity was not significantly changed by steroid treatment, but that E_2 may modulate the mechanism by which phorbol esters act to bring about PLA_2 activation.

5.4 DISCUSSION

The results presented in this Chapter suggest that E_2 may modulate LHRH priming by altering PKC isoform expression and action in gonadotrophs. The magnitudes of the priming response to LHRH and the secretory response to PDBu, measured *in vitro*, were seen to alter with the stage of the oestrous cycle (Table 5.1, Figure 5.1), consistent with previous results (Aiyer *et al,* 1974a, 1974b; Castro-Vazquez and McCann, 1975; Waring and Turgeon, 1980). This variation in anterior pituitary responsiveness to LHRH and PDBu correlated with changes in circulating levels of E_2 that are measured *in vivo*. Ovariectomised rat hemipituitaries did not exhibit the biphasic LH release response to LHRH which is characteristic of priming unless the animals had received prior treatment with E_2 (Table 5.2). These observations support a number of reports (Aiyer and Fink, 1974; Greeley *et al,* 1975; Fink and Henderson, 1977; Speight and Fink, 1981; Cover and Buckingham, 1989), and suggest that E_2 may confer on the gonadotroph at least one component of the capacity to prime.

Interestingly, both baseline and LHRH-induced LH release from E_2-treated ovariectomised rat tissue (Table 5.2) was several-fold greater than equivalent responses measured using tissue from intact female rats at any stage of the oestrous cycle (Table 5.1). There is evidence that the rat gonadotroph is maintained in a low state of responsiveness to LHRH by non-steroidal factors which are released from the ovary (Danforth *et al,* 1987; de Koning *et al,* 1987; Busbridge *et al,* 1990; Koppenaal *et al,* 1991). The absence of this inhibitory influence is suggested to account for the high rate of LH release that is observed following ovariectomy (van Dieten *et al,*

1989). Ovariectomised rat gonadotroph may, therefore, already exist in a state of increased responsiveness analogous to a 'primed' state, which may explain why a biphasic secretory response to LHRH was not observed in ovariectomised rat tissue. However, the 1st h LH release response to LHRH measured in ovariectomised rat tissue (Table 5.2) was not different from that observed using pro-oestrous rat tissue (Table 5.1), suggesting that there is no apparent increase in gonadotroph responsiveness to LHRH following ovariectomy. In fact, the 2nd h response to LHRH is reduced following ovariectomy, suggesting that the ovary can have a positive influence on gonadotroph responsiveness. Although these non-steroidal factors may not be essential for conferring the ability to prime on the gonadotroph, their negative actions may be important for controlling the magnitude of LHRH-induced gonadotrophin release and LHRH priming in steroid-'primed' animals (Culler, 1992).

The results presented here suggest that E_2 may have a positive influence on gonadotroph responsiveness to LHRH by having an effect on the activity and actions of the H7-resistant PKC, or PKC-like kinase, that is involved in the mechanism of LHRH priming. Preliminary evidence indicated that the enhanced 2nd h 'primed' response to LHRH measured in hemipituitaries from E_2-treated ovariectomised rats was inhibited by staurosporine, but not H7 (Table 5.2), although the statistical significance of this action could not be determined due to the small sample size. Nevertheless, these results do suggest that the enhanced 2nd h secretory response to LHRH, observed in tissue from E_2-treated ovariectomised rats, is dependent upon the actions of an H7-resistant, staurosporine-sensitive kinase. However, using hemipituitaries from steroid-untreated ovariectomised rats, 2nd h LHRH-induced LH release was also partially inhibited by staurosporine although a primed response to LHRH was not apparent (Table 5.2). Protein kinase C inhibitors can also block later responses to LHRH in dispersed anterior pituitary cells, cultured in E_2-free conditions, although they do not exhibit a secretory response to LHRH

which is typical of priming (Hirota *et al,* 1985; Chang *et al,* 1987b; Stojilkovic *et al,* 1991). It would seem, therefore, that PKC activation may be required, in part, for later responses to LHRH in both ovariectomised rat tissue and dispersed anterior pituitary cells. The involvement of PKC in later secretory responses to LHRH in ovariectomised rat tissue was not entirely unexpected since PKC activators can induce LH release from ovariectomised rat tissue by a staurosporine-sensitive (albeit an H7-resistant) process (Table 5.3). Thus, the H7-resistant PKC involved in LHRH priming may still have a role in LH release responses in ovariectomised rat tissue, but this is amplified upon treatment with E_2.

Consistent with a facilitatory effect of E_2 on gonadotroph PKC action, following E_2 treatment of ovariectomised rats, the magnitude of PDBu-induced LH release was greatly enhanced (Figure 5.2). Furthermore, the amount of PDBu-stimulated anterior pituitary cytosolic PKC activity was significantly greater in tissue from E_2-treated, compared to untreated ovariectomised rats (Table 5.4), supporting previous data (Drouva *et al,* 1990; Joubert-Bression *et al,* 1990) and an increase in pituitary PKC content was revealed by the [^{3}H]-PDBu binding studies (Table 5.6). These results, when considered together with evidence that the facilitatory action of E_2 on LH responses can be prevented by protein synthesis inhibitors (Debeljuk *et al,* 1978; Fahmy *et al,* 1989), suggest that E_2 enhances LH responses by inducing synthesis of additional PKC(s) molecules. These additional E_2-induced PKC(s) are pharmacologically distinct from those which mediated PKC-induced LH release prior to E_2 treatment (Figure 5.2, Table 5.3). That is, in tissue from untreated ovariectomised rats, those PKCs which induce LH release are activated by both PDBu and DOG, whereas, with E_2 treatment, there is the additional involvement of PDBu-sensitive but DOG-insensitive kinases. Correspondingly, in pro-oestrous rats, both DOG-insensitive, and DOG-activated kinases were also involved in the LH release response (Chapter 3). Protein kinase C(s) that are resistant to activation by DOG are

also involved in PDBu-induced GH release in from ovariectomised and pro-oestrous rat tissue (Figure 5.3, Chapter 3).

The PKCs which mediate PKC activator-induced LH and GH release from ovariectomised rat hemipituitaries are relatively resistant to H7 (Table 5.3). Correspondingly, in anterior pituitary cytosol from ovariectomised rats, a PDBu-induced, Ca^{2+}-independent component of PKC activity was detected which was relatively H7-resistant. Following E_2 treatment, the amount of Ca^{2+}-independent, H7-resistant PKC activity was increased (Figure 5.4, Table 5.5), the LH release response to PDBu was also increased (Table 5.3) and an H7-resistant LHRH priming-like effect was observed using E_2-treated ovariectomised rat tissue (Table 5.2). Thus, E_2 treatment may enhance the activity and actions of the H7-resistant PKC(s) that is involved in LHRH priming and PKC activator-induced LH release.

In contrast to the reduced potency of H7 on anterior pituitary Ca^{2+}-independent PKC activity, PDBu-elicited, Ca^{2+}-dependent and -independent PKC activities measured in cytosol from ovariectomised rat hypothalamus and hippocampus had the expected sensitivities to H7. The IC_{50} values for the inhibition of Ca^{2+}-dependent and -independent activities by H7 in ovariectomised rat hypothalamus cytosol were 18 ± 8 μM and 14 ± 4 μM (means ± SEM, n = 4), respectively. Corresponding IC_{50} values for H7 measured in hippocampus cytosol were 20 ± 4 μM and 21 ± 1 μM (n = 4) for Ca^{2+}-dependent and -independent activities respectively. Oestrogen treatment had no significant effect upon the H7 sensitivity of PDBu-induced PKC activity in either hippocampus or hypothalamus.

Interestingly, E_2 treatment of ovariectomised rats also enhanced the amount of Ca^{2+}-dependent, H7-sensitive, PDBu-induced PKC activity in anterior pituitary cytosol (Tables 5.4 and 5.5), suggesting that E_2 treatment may facilitate the actions of other PKC(s) in addition to those involved on LHRH priming and PDBu-induced LH release. Although a component of PDBu-induced LH release from pro-oestrous rat tissue was readily blocked by H7, the LH release response to PDBu in

tissue from ovariectomised rats with or without E_2 treatment showed no obvious sensitivity to H7 (Table 5.3). Ovarian factors other than E_2 may influence the involvement of the H7-sensitive PKCs in the mechanism of phorbol ester-induced LH release. For example, progesterone can further enhance the actions of E_2 on LHRH-induced gonadotrophin release (Mann and Barraclough, 1973; Turgeon and Waring, 1981, 1990) suggesting that this steroid may also regulate gonadotroph responsiveness and may possibly influence the activity of the H7-sensitive PKCs. Androgens can have inhibitory effects on LHRH-induced LH release in cultured cells *in vitro* (Drouin and Labrie, 1975; Tibolt and Childs, 1985). Testosterone replacement of castrated male rats apparently restores LHRH priming *in vivo* not by facilitating later responses to LHRH as is the case for E_2, but by reducing the initial response to LHRH (Nazian, 1986). Thus, in the intact rat, a combination of steroidal and non-steroidal gonadal factors may be responsible for controlling gonadotroph responsiveness. Clearly, additional studies are required to ascertain the effect of other gonadal factors on the cellular mechanisms which induce priming and on the actions of the H7-sensitive PKC form(s) involved in PDBu-induced LH release. Dispersed anterior pituitary cells, maintained in culture, may prove to be a useful model for such future studies since culture conditions can be easily defined and manipulated, unlike the whole animal model which has been used in the present experiments. It will be of interest to alter the culture conditions to mimic the levels and combination of gonadal steroids, non-steroidal factors and LHRH pulses to which the pituitary is exposed on pro-oestrus and to examine their effect on the LHRH priming mechanism. However, the dispersed cell model of anterior pituitary function can suffer from a number of drawbacks. Enzymatic dispersion can change in receptor characteristics and cause cell membrane damage (Naor *et al,* 1980) which will affect gonadotroph responsiveness. In addition, there is evidence that a specific cellular association exists between lactotrophs and gonadotrophs in rat anterior pituitary (Nakane, 1970; Fletcher *et al,* 1975) and that physiological interactions may occur between these cell

types (Denef and Andries, 1983). The secretory profiles of dispersed cells may be altered due to disruption of these interactions between gonadotrophs and mammotrophs.

Oestrogen may have modulatory actions on several factors that can be involved in cellular signalling mechanisms. For example, E_2 treatment of rat anterior pituitary cell cultures can enhance LH release in response to the voltage-sensitive Ca^{2+} channel agonist, BAY K 8644, suggesting that E_2 treatment can facilitate some means of activating gonadotroph voltage-sensitive Ca^{2+} channels (Drouva *et al,* 1988). In addition, E_2 treatment of long-term ovariectomised ewes and of ovine anterior pituitary cell cultures can cause a long-term increase in pituitary LHRH receptor number (Gregg and Nett, 1989; Laws *et al,* 1990). In the rat ventromedial hypothalamus, E_2 treatment can also induce the synthesis of a characteristic 70 kDa protein of yet unspecified function (Mobbs *et al,* 1991). Therefore, it is likely that E_2 modulation of cellular signalling within gonadotrophs can occur at sites other than PKC and thereby contribute to the facilitatory actions of E_2 on gonadotrophin release. It is possible that the modulatory effect of E_2 on these factors may, in part, contribute to the change in the time course of PDBu-induced LH release observed in ovariectomised rat tissue after E_2 treatment.

In some *in vitro* models, E_2 treatment has positive effects on LH-β gene transcription (Shupnik *et al,* 1989). Since pituitary and serum concentrations of LH-β subunit mRNA correlate with pituitary content of LH (Fetherston and Boime, 1982), this action of E_2 may have positive effects on LH pool size. However, *in vivo* replacement of E_2 in long-term ovariectomised animals (as here) seems instead to prevent the increase of LH-β mRNA levels that occurs following ovariectomy (Counis *et al,* 1983; Gharib *et al,* 1988; Papavisiliou *et al,* 1986). Furthermore, there is no change in total pituitary LH content in long-term E_2-treated ovariectomised rats (Marchetti *et al,* 1982). Instead, E_2 can enhance LHRH-induced LH synthesis and glycosylation, as assessed by the incorporation of radiolabelled amino acids or

glucosamine respectively, into LH released from cultured cells (Liu and Jackson, 1977; Tang, 1980; Ramey *et al,* 1987; Liu and Jackson, 1990). However, the experiments here with ionomycin (Figure 5.5) indicated that the facilitatory actions of E_2 on phorbol ester-induced LH secretion are not the result of an E_2-induced increase in releasable gonadotrophin content prior to a stimulus. It is, of course, possible that Ca^{2+}-independent release processes or release mechanisms involving factors additional to Ca^{2+}, such as GTP-dependent processes, could be modified by E_2 treatment. However, these effects of E_2 may remain undetected in these experiments.

Although the induction of LHRH priming is dependent upon PLA_2 activation down-stream of PKC action (Chapter 4), and cycle-dependent changes in the involvement of PLA_2 in the mechanism of PKC-induced LH release were observed (Figure 5.6), the experiments described here suggest that E_2 does not directly influence anterior pituitary PLA_2. The magnitude of ionomycin-induced [^{3}H]-AA release from dispersed rat anterior pituitary cells was not altered by E_2 treatment suggesting that this steroid does not directly modulate PLA_2 activity elicited by directly raising intracellular Ca^{2+} levels (Rosenthal *et al,* 1989) (Table 5.8). These results are in agreement with a previous report which showed that neither PLA_2 nor melittin-induced LH release from anterior pituitary cell cultures was altered by E_2 treatment (Liu and Jackson, 1989). It would seem, however, that E_2 treatment may have an effect on the cellular mechanisms which control gonadotroph PLA_2 activity. In anterior pituitary tissue from ovariectomised rats, PDBu-induced LH release was not altered by the PLA_2 inhibitor, quinacrine (Table 5.7), suggesting that the PKC(s) involved in this response do not target PLA_2, unlike the pro-oestrous rat model of gonadotroph function (Figure 5.6, Chapter 4). In agreement with this hypothesis, PDBu was unable to elicit a significant increase in [^{3}H]-AA release from pre-labelled ovariectomised rat anterior pituitary tissue or from cells maintained under E_2-free culture conditions (Tables 5.8 and 5.9). In contrast, [^{3}H]-AA release from E_2-treated pre-labelled cells or from tissue removed from E_2-treated ovariectomised rats

was significantly increased by PDBu (Tables 5.8 and 5.9). In addition, PDBu-induced LH release from E_2-treated, ovariectomised rat tissue was sensitive to quinacrine (Table 5.7). These results suggest that E_2 may have an indirect influence on the actions of PLA_2 in gonadotroph secretory responses perhaps by modulating the actions and activity of the PKC(s), or some other intermediate step which is involved in the control of anterior pituitary PLA_2 activity. In contrast, acute progesterone treatment of E_2 pre-treated cells can enhance melittin-induced LH release (Ortmann *et al,* 1992) indicating that progesterone may either have a direct modulatory influence on PLA_2 action in gonadotrophs, or an action on some process subsequent to PLA_2 activation.

Some studies have suggested that gonadectomy or steroid treatment can alter GH release patterns measured in the female rat *in vivo* (Jansson *et al,* 1984). However, in the model described here, E_2 did not regulate either PKC actions on GH release (Figure 5.3) or the size of the releasable pool of GH (Figure 5.5) and E_2 treatment of ovariectomised rats had no effect on either pituitary GH synthesis or mRNA content (Shupnik *et al,* 1979). Furthermore, there appeared to be no clear relationship between GH secretory patterns in intact female rats and the stage of the oestrous cycle *in vivo* (Clark *et al,* 1987b) and *in vitro* (Figure 5.1), consistent with our evidence which suggests that E_2 does not play an important role in modulating anterior pituitary gland responsiveness with respect to PKC-mediated GH release. Oestrogen treatment of cultured pituitary cells *in vitro* has correspondingly been shown to enhance phorbol ester-induced LH, but not GH, release (Drouva *et al,* 1990). It is likely, therefore, that action of E_2 on GH secretory patterns described by Jansson and co-workers (1984) may be due to an action of the steroid at a site other than the pituitary, perhaps at the level of the hypothalamus or at other regions of the CNS. Quinacrine was unable to inhibit PDBu-induced GH release from either E_2-treated or -untreated ovariectomised rat tissue (Table 5.7), suggesting that PLA_2 is not

involved in the mechanism of PDBu-induced GH release, supporting the observations made using tissue from intact rats (Chapter 4, Figure 5.7).

The PKC isoforms which have been sequenced (α, β_I, β_{II}, γ, δ, ε, ζ, η , θ and λ) have distinct tissue distributions (Nishizuka, 1988; Osada *et al,* 1990; Y. Nishizuka, personal communication), indicating that the expression of each isoform is differentially regulated in different tissues. However, little is known about the effects of gonadal steroids on the expression of each isoform. Androgens may modulate the synthesis of the α, and to a lesser extent, the β PKC isoform in the male rat prostate (Goueli, 1990). Since both Ca^{2+}-dependent and -independent PKC activities were increased by E_2 treatment, it seems that other PKCs may be induced by E_2, in addition to the Ca^{2+}-independent, H7-resistant PKC relevant here in the increased responsiveness of gonadotrophs. Although the E_2-induced H7-resistant PKC would initially appear to be a B series (or related) isoform, this kinase may represent a modified A series isoform since it is clear that the classification of the known PKC isoforms according to their Ca^{2+}-dependency may be less clear cut than when originally conceived (see Chapter 3, section 3.4 for further discussion). Data obtained using highly purified expressed recombinant protein have shown that the δ and ε isoforms of PKC are more sensitive to H7 (Schaap and Parker, 1990; Uchida *et al,* 1991) than those E_2-induced forms that mediate LH and GH release in this model, suggesting that neither δ nor ε PKC is significantly involved in the release of these hormones in tissue from ovariectomised rats. Furthermore, the ζ and λ PKC isoforms are reported to be unresponsive to phorbol esters (Ono *et al,* 1989, Y. Nishizuka, personal communication), suggesting that neither of these PKC isoforms represent the E_2-induced PKC. It would seem, therefore, that the E_2-induced PKC isoform(s) which mediate PDBu-induced LH release from ovariectomised rat anterior pituitary tissue and that is involved in LHRH priming, may be a modified form of a known PKC isoform, a poorly characterised Ca^{2+}-independent isoform, such as η or θ or an isoform of PKC (or closely related protein kinase) which has not yet been sequenced.

Recently, antibodies for several of the known isoforms of PKC have become commercially available such that Western blot analysis may be able to reveal which isoforms are induced by steroid treatment. Alternatively, Northern blot studies, using mRNA probes with sequences specific to different PKC isoforms, could be used to identify the PKC isoforms that are induced by E_2 treatment. In addition, further experiments are required to confirm that the E_2-induced PKC(s) involved in PDBu responses are the same as those involved in LHRH priming. Following identification of the E_2-induced PKC isoform(s) using the methods described above, it may be possible to use either selective antisense oligonucleotide sequences to this PKC or specific antibodies in permeabilised cells to estimate the constitution of this enzyme to LHRH priming.

FIGURE 5.1

The effect of phorbol 12,13-dibutyrate (PDBu) on LH and GH release from female rat hemipituitaries removed on different stages of the oestrous cycle

Hemipituitaries were incubated for a basal h with no drug, followed by consecutive hours in the presence of PDBu (300 nM) and both LH (●) and GH (■) release were determined. Each value represents the mean ± SEM for 4 - 10 determinations.

Figure 5.1

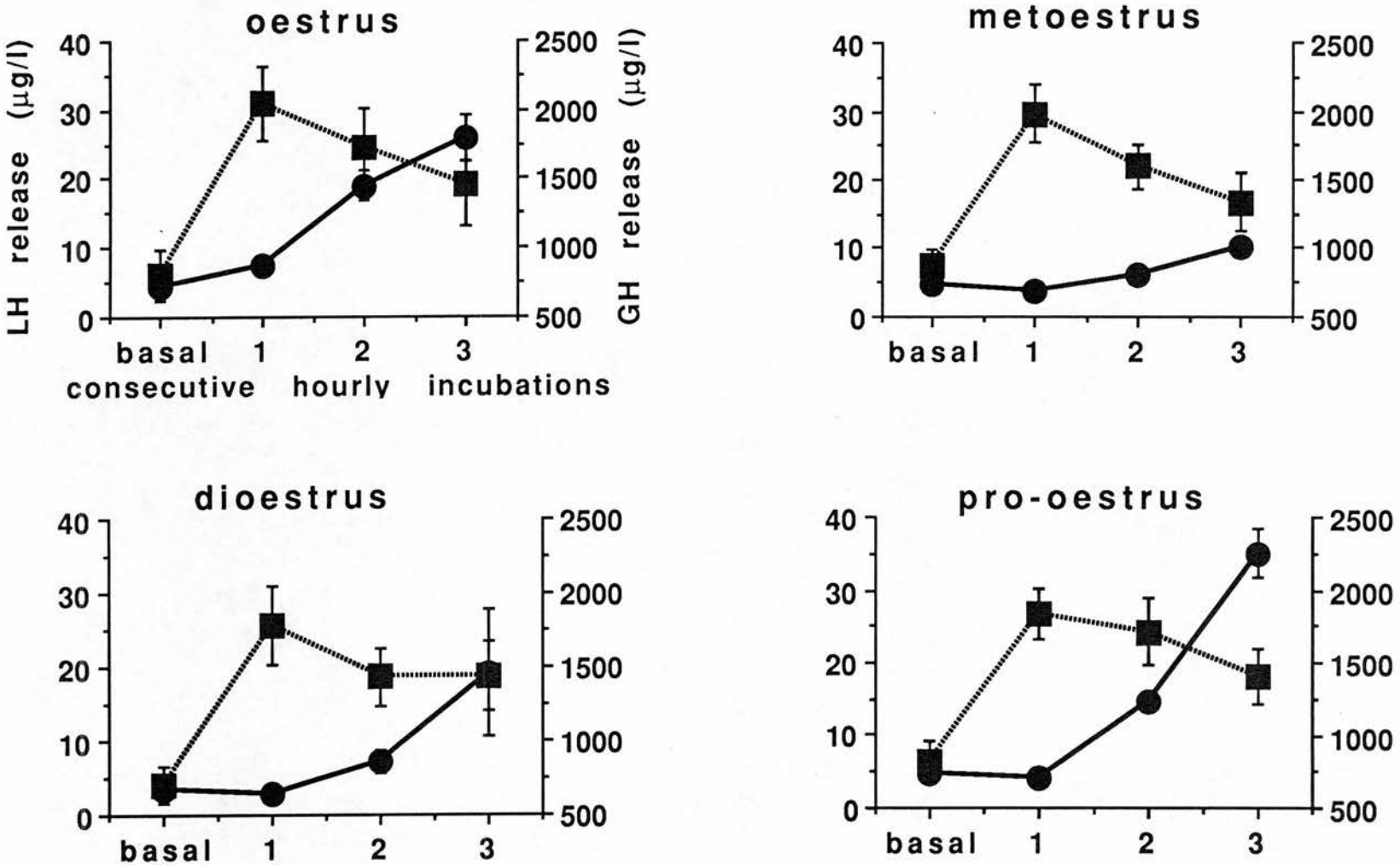

oestrus
LH release (µg/l)
GH release (µg/l)
40
30
20
10
0
2500
2000
1500
1000
500
basal
1
2
3
consecutive
hourly
incubations
metoestrus
basal
1
2
3
dioestrus
basal
1
2
3
pro-oestrus
basal
1
2
3

FIGURE 5.2

The effect of (a) phorbol 12,13-dibutyrate (PDBu) and (b) 1,2-dioctanoyl-*sn*-glycerol (DOG) on LH release from anterior pituitary tissue taken from oestrogen (E_2)-treated and untreated ovariectomised (OVX) rats

Tissue removed from ovariectomised rats with (hatched bars) or without (open bars) E_2 treatment was incubated with medium only in the basal h followed by 3 consecutive hourly incubations (1st, 2nd, 3rd h) in the presence of either (a) 300 nM PDBu or (b) 200 μM DOG. The data represent net hormone release, i.e. baseline release was subtracted. Luteinizing hormone release from both E_2-treated and -untreated tissue was increased above baseline levels during all hours of incubation with either PDBu or DOG. Phorbol 12,13-dibutyrate- but not DOG-induced LH release was significantly enhanced by E_2 treatment ($^*p < 0.05$, $^{**}p < 0.01$ comparing E_2-treated tissue with E_2-untreated tissue, Mann-Whitney U-test). Values are means ± SEM (n = 5 - 10).

Figure 5.2

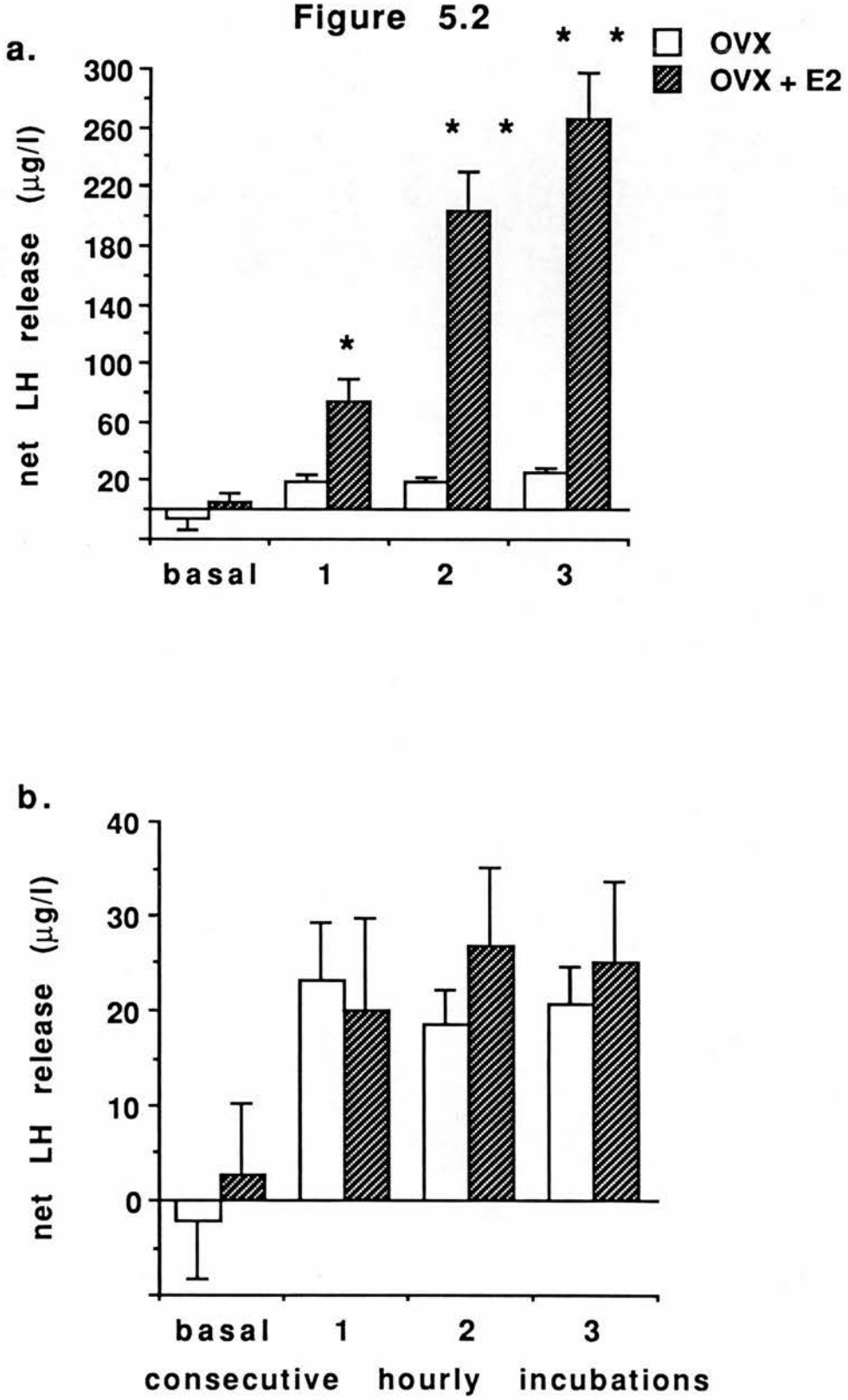

a.
net LH release (µg/l)
300
260
220
180
140
100
60
20
OVX
OVX + E2
*
*
*
*
*
basal
1
2
3
b.
net LH release (µg/l)
40
30
20
10
0
basal
1
2
3
consecutive
hourly
incubations

FIGURE 5.3

The effect of (a) phorbol 12,13-dibutyrate (PDBu) and (b) 1,2-dioctanoyl-*sn*-glycerol (DOG) on GH release from anterior pituitary tissue taken from oestrogen (E_2)-treated and -untreated ovariectomised (OVX) rats

Tissue was removed from ovariectomised rats which had received either E_2 treatment (hatched bars) or no steroid (open bars) and was incubated with medium only in the basal h followed by 3 consecutive hourly incubations (1st, 2nd, 3rd h) in the presence of either 300 nM PDBu or 200 μM DOG. Growth hormone release was increased in the presence of PDBu, but not DOG ($p < 0.05$, Duncan's New Multiple Range test). There was no significant difference in the magnitude of GH release between E_2-treated and -untreated tissue (Mann-Whitney U-test). Values are means ± SEM (n = 4 - 10).

Figure 5.3

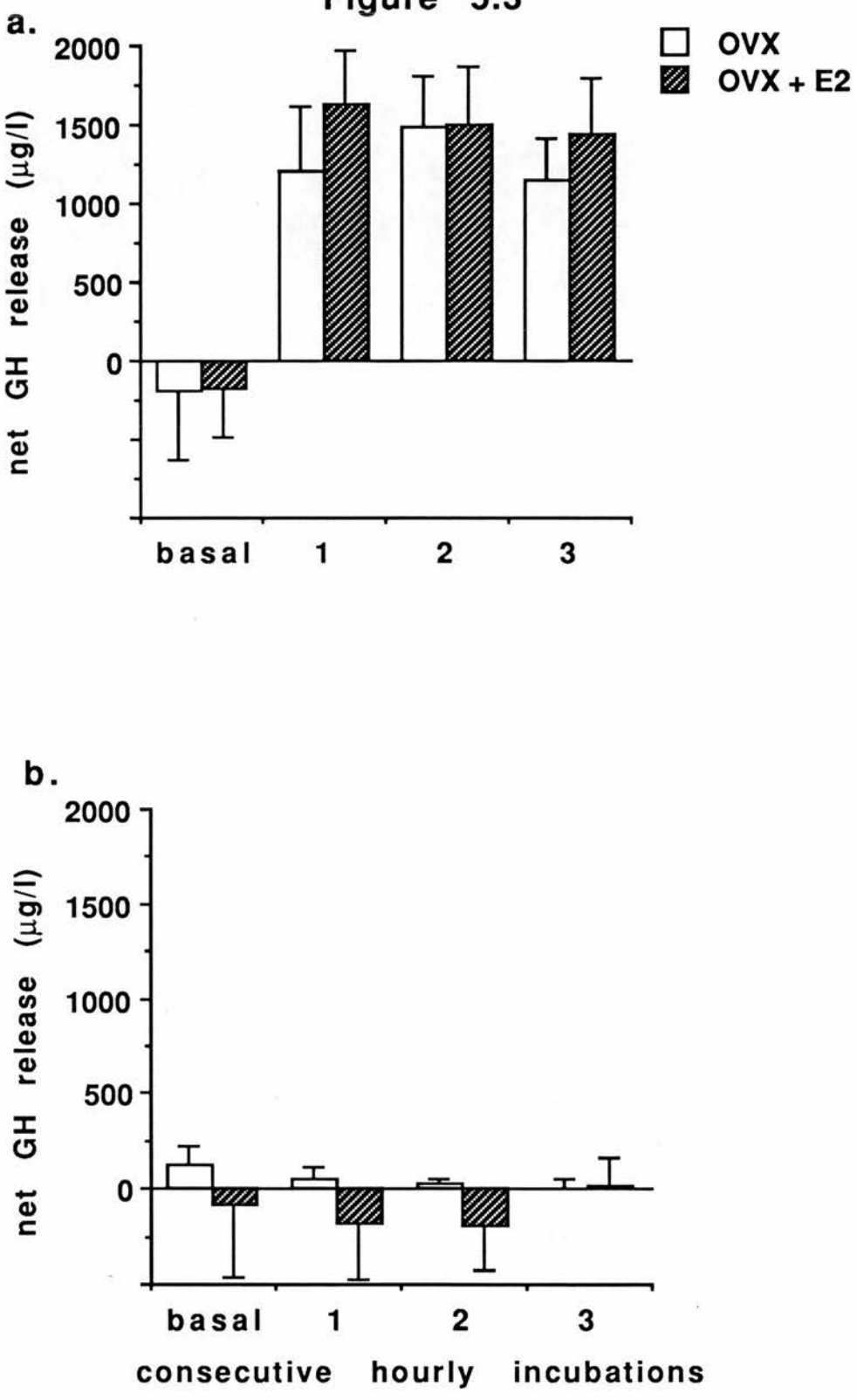

a.
2000
1500
1000
500
0
net GH release (µg/l)
OVX
OVX + E2
basal
1
2
3
b.
2000
1500
1000
500
0
net GH release (µg/l)
basal
1
2
3
consecutive
hourly
incubations

FIGURE 5.4

The effect of H7 on phorbol 12,13-dibutyrate (PDBu) -stimulated Ca^{2+}-independent and Ca^{2+}-dependent cytosolic PKC activity from ovariectomised (OVX) rat anterior pituitary tissue with or without oestrogen (E_2) treatment

Anterior pituitary tissue was removed from ovariectomised rats which had either received E_2 replacement (closed symbols) or no steroid (open symbols). Cytosolic PKC activity was partially purified and the phosphatidylserine-dependent, histone IIIS kinase activity measured with 1 μM PDBu and various concentrations of H7 in the presence (100 μM) (○/●) or absence (< 3 nM) (□/■) of Ca^{2+}. Calcium-independent, PDBu-induced PKC activity in ovariectomised rat anterior pituitary cytosol was less sensitive to H7 block than Ca^{2+}-dependent activity. This profile of H7-sensitivity was not significantly altered by E_2 treatment. Each point on the curve is the mean ± SEM of 4 - 6 determinations.

Figure 5.4

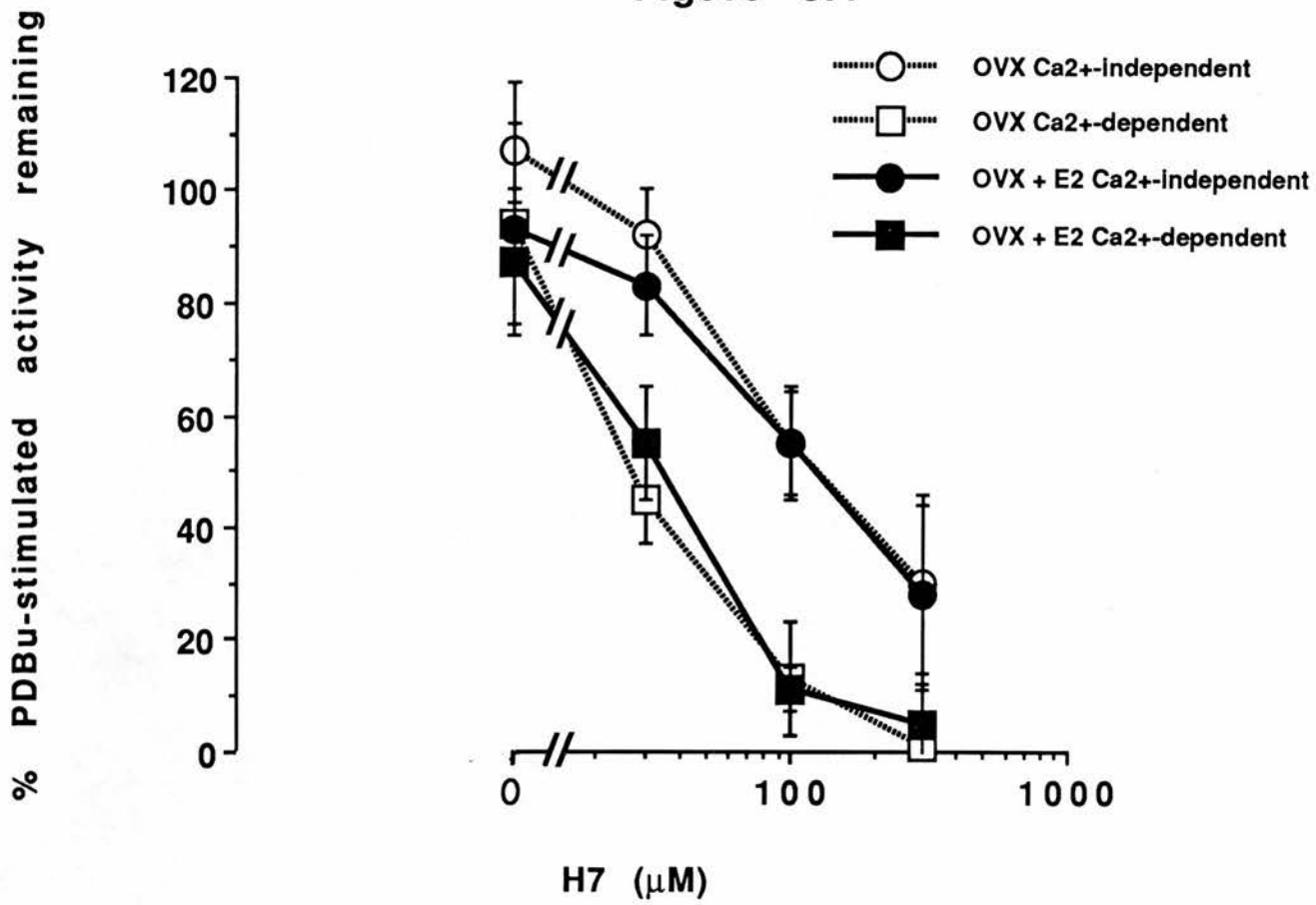

% PDBu-stimulated activity remaining
120
100
80
60
40
20
0
0
100
1000
H7 (μM)
OVX Ca2+-independent
OVX Ca2+-dependent
OVX + E2 Ca2+-independent
OVX + E2 Ca2+-dependent

FIGURE 5.5

The effect of ionomycin on (a) LH and (b) GH release from anterior pituitary tissue taken from oestrogen (E_2)-treated and -untreated ovariectomised (OVX) rats

Tissue was removed from ovariectomised rats which had received either E_2 treatment (hatched bars) or no steroid (open bars) and was incubated with medium only in the basal h followed by 3 consecutive hours in the presence of 30 μM ionomycin. The data represents net hormone release, i.e. baseline release was subtracted. Release of both LH and GH in the presence of ionomycin was not significantly altered by E_2 treatment (Student's t-test). Values are mean ± SEM (n = 4).

Figure 5.5

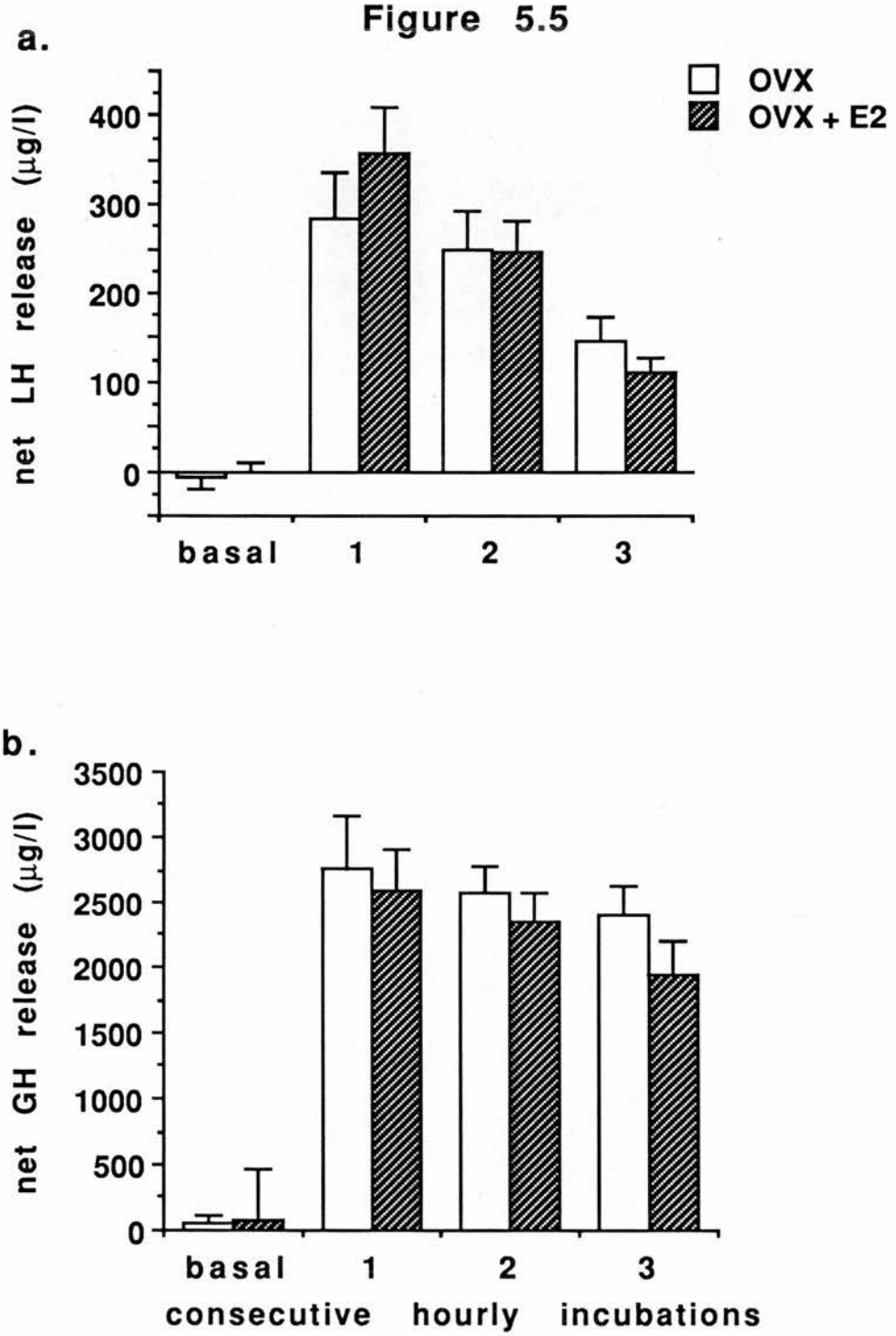

a.
net LH release (µg/l)
400
300
200
100
0
OVX
OVX + E2
basal
1
2
3
b.
net GH release (µg/l)
3500
3000
2500
2000
1500
1000
500
0
basal
1
2
3
consecutive
hourly
incubations

FIGURE 5.6

The effect of quinacrine on phorbol 12,13-dibutyrate (PDBu)-induced LH release from female rat hemipituitaries removed at different stages of the oestrous cycle

Hemipituitaries were incubated for a basal h with either no drug (open bars) or with quinacrine (50 μM) (hatched bars), followed by consecutive hours in the additional presence of PDBu (300 nM). Data was calculated as a fold of basal h release. The statistical significance of the inhibitory effect of quinacrine was determined (* $p < 0.05$, Mann-Whitney U-test). Each value represents the mean ± SEM for 4 - 10 determinations.

Figure 5.6

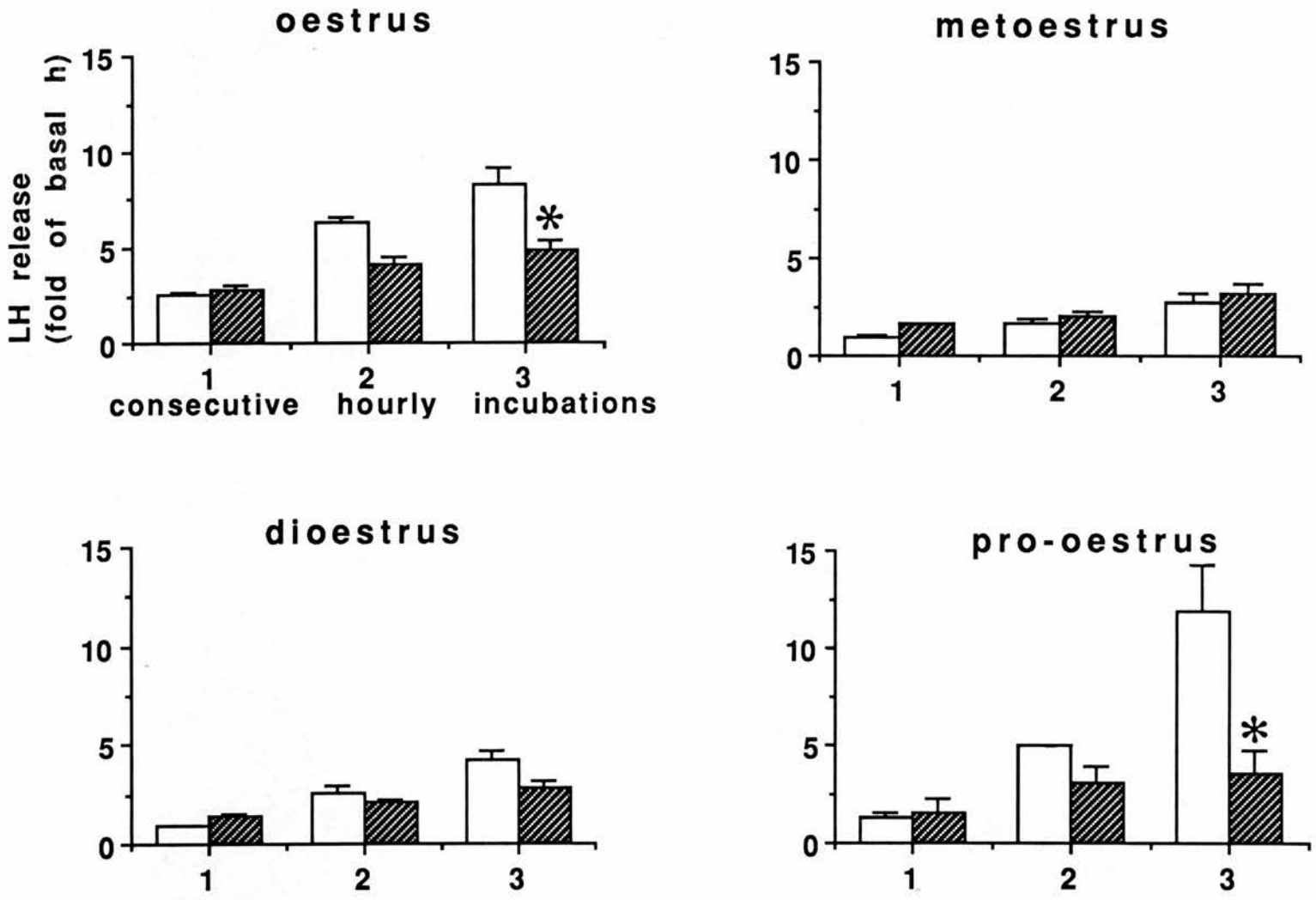

oestrus
metoestrus
dioestrus
pro-oestrus
LH release
(fold of basal h)
15
10
5
0
1
2
3
consecutive
hourly
incubations
*

FIGURE 5.7

The effect of quinacrine on phorbol 12,13-dibutyrate (PDBu)-induced GH release from female rat hemipituitaries removed at different stages of the oestrous cycle

Hemipituitaries were incubated for a basal h with either no drug (open bars) or with quinacrine (50 μM) (hatched bars), followed by consecutive hours in the additional presence of PDBu (300 nM). Quinacrine had no significant inhibitory effect on GH release measured at any hour on any stage of the oestrous cycle. Each value represents the mean ± SEM for 4 - 10 determinations.

Figure 5.7

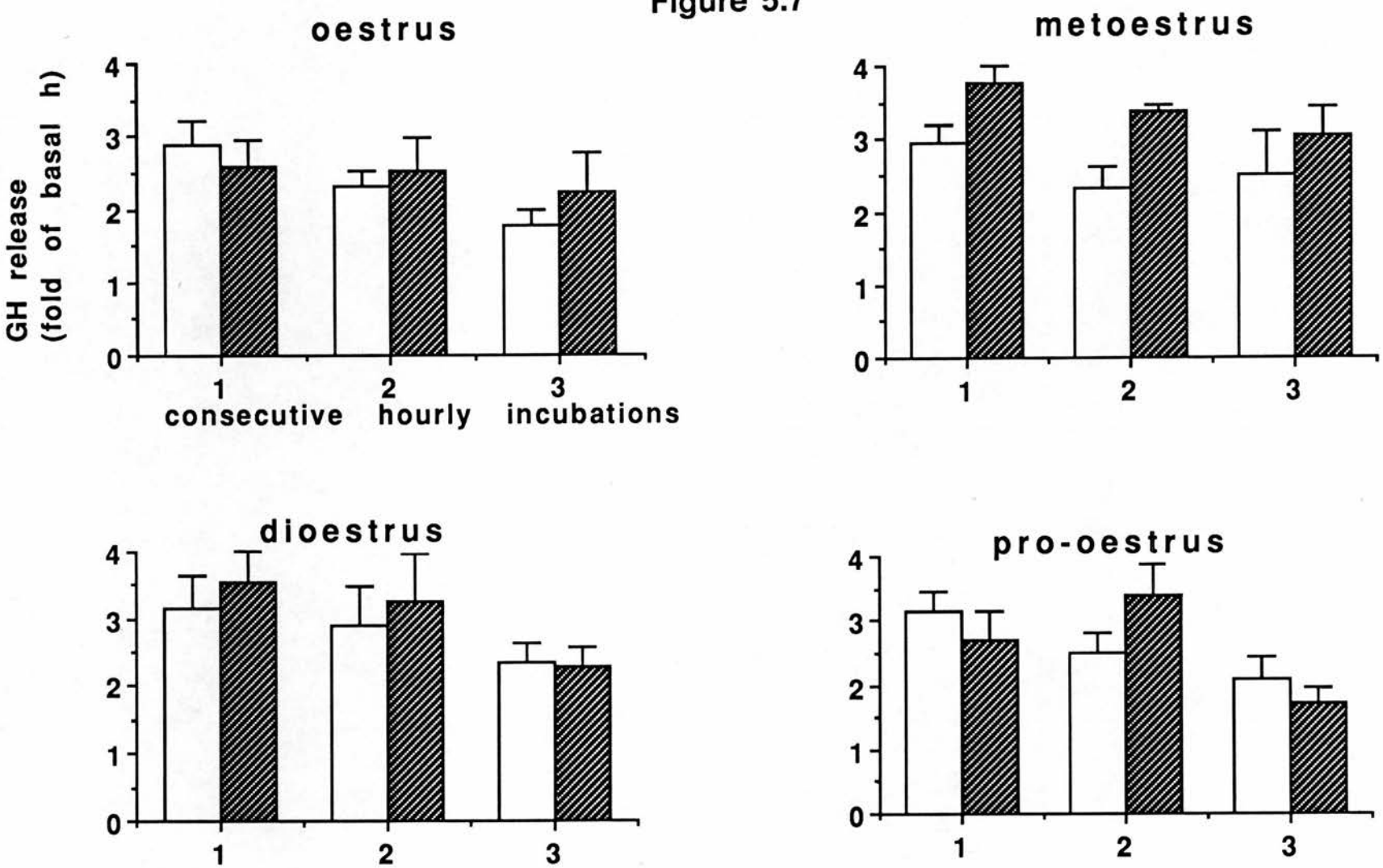

oestrus
metoestrus
dioestrus
pro-oestrus
GH release
(fold of basal h)
0
1
2
3
4
1
consecutive
2
hourly
3
incubations

TABLE 5.1

The effect of staurosporine on LHRH-induced LH release over the days of the rat oestrous cycle

Hemipituitaries were incubated *in vitro* for 3 consecutive hours. In the basal h either medium alone (controls) or staurosporine (300 nM) was present. In the following 2 h there was, in addition, LHRH (1 nM). The statistical significance of the inhibitory effect of staurosporine was determined, *$p < 0.05$, **$p < 0.01$, Student's t-test. Values given are the mean ± SEM (number of determinations in parentheses).

		LH release (μg/l)			
Days of cycle		**basal h**	**1st h**	**2nd h**	**n**
oestrus	control	8.4 ± 1.6	14.2 ± 1.7	35.9 ± 2.3	4
	staurosporine	6.2 ± 0.7	9.5 ± 1.1	9.2 ± 1.1*	4
metoestrus	control	4.9 ± 1.2	5.3 ± 0.4	25.1 ± 2.2	4
	staurosporine	6.1 ± 1.4	4.6 ± 1.0	5.8 ± 1.3*	4
dioestrus	control	5.3 ± 1.2	8.6 ± 1.0	55.7 ± 4.9	6
	staurosporine	2.9 ± 0.6	4.6 ± 0.4	6.2 ± 0.6**	6
pro-oestrus	control	5.3 ± 0.7	27.0 ± 2.6	159.7 ± 14.4	6
	staurosporine	6.6 ± 0.8	25.4 ± 3.0	30.7 ± 4.3**	6

TABLE 5.2

The effect of PKC inhibitors on LHRH-induced LH release from anterior pituitary tissue taken from ovariectomised (OVX) rats with or without oestrogen (E_2) treatment

Hemipituitaries were incubated *in vitro* for 3 consecutive hours. In the basal h there was either medium only or staurosporine (300 nM) or H7 (30 μM). In the 1st h and 2nd h there was, in addition, LHRH (1 nM) or no drug (baseline). The statistical significance of the effects caused by PKC inhibitors is indicated by * ($p < 0.05$, Mann-Whitney U-test). All values are mean ± SEM (number of determinations in parentheses).

	LH release (μg/l)		
	basal h	**1st h**	**2nd h**
	OVX		
baseline	20.6 ± 1.8 (4)	13.3 ± 2.5 (4)	10.0 ± 1.3 (4)
LHRH	18.3 ± 2.1 (9)	28.4 ± 3.2 (9)	34.9 ± 7.6 (9)
LHRH + staurosporine	21.7 ± 2.5 (11)	28.0 ± 2.8 (11)	*19.9 ± 2.5 (10)
LHRH + H7	20.5 ± 7.5 (3)	33.8 ± 3.7 (3)	40.9 ± 5.7 (3)
	OVX + E_2		
baseline	36.3 ± 3.0 (4)	34.8 ± 5.2 (4)	22.9 ±7.8 (4)
LHRH	40.7 ± 5.5 (3)	270.3 ± 2.8 (3)	447.9 ± 4.1 (3)
LHRH + staurosporine	39.6 ± 7.2 (3)	219.9 ± 13.0 (3)	306.0 ± 30.2 (3)
LHRH + H7	39.9 ± 7.1 (3)	290.2 ± 12.0 (3)	535.1 ± 19.6 (3)

TABLE 5.3

The effect of PKC inhibitors on PKC activator-induced LH and GH release from anterior pituitary tissue taken from ovariectomised (OVX) rats with or without oestrogen (E_2) treatment

Hemipituitaries were incubated *in vitro* for 4 consecutive hours. In the basal h there was either medium only or staurosporine (300 nM) or H7 (30 μM). In 1st, 2nd and 3rd h there was, in addition, either DOG (200 μM) or PDBu (300 nM). The data show net LH release (i.e. with baseline subtracted) measured in the 3rd h and net GH release measured in the 1st h. All values are mean ± SEM (number of determinations in parentheses). The statistical significance of the effects caused by PKC inhibitors is indicated by * ($p < 0.05$, Mann-Whitney U-test).

	LH release (μg/l)	
	OVX-untreated	**E_2-treated**
PDBu	30.9 ± 4.1 (10)	308.0 ± 35.1 (6)
PDBu + staurosporine	*8.0 ± 4.6 (8)	*94.6 ± 18.4 (8)
PDBu + H7	29.8 ± 4.8 (6)	328.5 ± 25.2 (6)
DOG	22.0 ± 2.9 (5)	24.9 ± 2.8 (8)
DOG + staurosporine	*8.9 ± 3.0 (4)	*9.9 ± 4.4 (4)
DOG + H7	24.7 ± 2.9 (6)	21.9 ± 7.6 (6)
	GH release (μg/l)	
PDBu	1828.5 ± 256.6 (6)	1871.7 ± 290.1 (10)
PDBu + staurosporine	*138.5 ± 56.9 (6)	*240.8 ± 184.6 (5)
PDBu + H7	2079.2 ± 374.5 (6)	1866.8 ± 312.2 (5)

TABLE 5.4

Phorbol 12,13-dibutyrate (PDBu)- and 1,2-dioctanoyl-*sn*-glycerol (DOG)-stimulated PKC activity in anterior pituitary cytosol from ovariectomised (OVX) rats with or without oestrogen (E_2) treatment

These data show the PKC-activator-evoked phosphatidylserine-dependent, histone IIIS kinase activity, measured in the presence of either PDBu (1 μM) or DOG (100 μM) at either < 3 nM or 100 nM free Ca^{2+}. All data are shown as mean ± SEM of 5 - 7 determinations, *$p < 0.05$ compared with the effect of E_2 on activator-stimulated PKC activity (Mann-Whitney U-test).

Activator-induced kinase activity (pmol thiophosphate/mg protein/15 min)		OVX	OVX + E_2-treated
PDBu	Ca^{2+}-independent	3056 ± 551	*4829 ± 420
	Ca^{2+}-dependent	2806 ± 651	*4535 ± 714
DOG	Ca^{2+}-independent	95 ± 36	138 ± 18
	Ca^{2+}-dependent	398 ± 26	*807 ± 17

TABLE 5.5

The IC_{50} values for H7 and staurosporine on phorbol 12,13-dibutyrate (PDBu)-induced PKC activity in anterior pituitary cytosol from ovariectomised (OVX) rats with or without oestrogen (E_2) treatment

Cytosolic PKC in anterior pituitaries removed from ovariectomised rats, with or without E_2 treatment, was partially-purified and the phosphatidylserine-dependent, histone IIIS activity was measured, using 1 μM PDBu as a PKC activator. Kinase activity was assayed either in the absence (< 3 nM) or presence (100 μM) of Ca^{2+}. The IC_{50} values for H7 and staurosporine were determined for the Ca^{2+}-independent activity and for the incremental activity induced by Ca^{2+}. Each point on the curves from which the IC_{50} values were derived was the mean ± SEM of 4 - 6 determinations.

	IC_{50} values for H7 (μM)	
	Ca^{2+}-independent	**Ca^{2+}-dependent**
OVX	104 ± 23	27 ± 4
OVX + E_2	113 ± 25	32 ± 3
	IC_{50} values for staurosporine (nM)	
OVX	132 ± 46	139 ± 65
OVX + E_2	137 ± 51	100 ± 10

TABLE 5.6

The number and affinity of specific [^{3}H]-phorbol 12,13-dibutyrate (PDBu) binding sites in anterior pituitary cytosol from ovariectomised (OVX) rats with and without oestrogen (E_2) treatment

Non linear, direct fitting Scatchard analysis of the data (with error weighting) was carried out using the programme, P.fit (Biosoft, Cambridge, UK). All data are shown as mean ± SEM, n = 4.

	E_2-untreated	**E_2-treated**
Maximum number of specific [^{3}H]-PDBu binding sites (pmol/mg protein)	8.3 ± 0.6	18.7 ± 1.4
[^{3}H]-PDBu binding site dissociation constant (nM)	15.7 ± 3.4	12.6 ± 0.8

TABLE 5.7

The effect of quinacrine on phorbol 12,13-dibutyrate (PDBu)-induced LH and GH release from anterior pituitary tissue removed from oestrogen (E_2)-treated and -untreated ovariectomised (OVX) rats

Hemipituitaries were incubated *in vitro* for 4 consecutive hours. In the basal h there was either medium only or quinacrine (50 μM). In the 1st, 2nd and 3rd h there was, in addition, PDBu (300 nM). The data shows net LH release measured in the 3rd h and net GH release measured in the 1st h of PDBu incubation. All values are mean ± SEM (number of determinations in parentheses). The statistical significance of the effects caused by quinacrine is indicated by * ($p < 0.05$, Mann-Whitney U-test).

	net LH release (μg/l)	
	OVX	**OVX + E_2**
PDBu	28.6 ± 5.4 (6)	431.7 ± 45.1 (6)
PDBu + quinacrine	32.9 ± 5.2 (6)	* 247.3 ± 36.7 (6)
	net GH release (μg/l)	
PDBu	2540.9 ± 347.0 (6)	2271.6 ± 199.5 (6)
PDBu + quinacrine	3105.0 ± 295.1 (4)	2761.4 ± 409.1 (4)

TABLE 5.8

The effect of oestrogen (E_2) treatment on phorbol 12,13-dibutyrate (PDBu)-induced [^{3}H]-arachidonic acid (AA) release from ovariectomised (OVX) rat anterior pituitary tissue

Pre-labelled pairs of pituitary quarters were incubated for 15 minutes either in medium containing no drug (basal) or PDBu (300 nM). The statistical significance of the effects caused by PDBu incubation compared to baseline is indicated by * ($p < 0.05$, Mann-Whitney U-test). Data are means ± SEM for the number of determinations shown in parentheses.

	[^{3}H]-AA release (% of total label incorporated)	
	OVX	**OVX + E_2**
baseline	1.46 ± 0.08 (6)	1.43 ± 0.04 (6)
PDBu	1.35 ± 0.07 (8)	*1.74 ± 0.04 (6)

TABLE 5.9

The effect of oestrogen (E_2) treatment on phorbol 12,13-dibutyrate (PDBu)- and ionomycin-induced [^{3}H]-arachidonic acid (AA) release from 5 day cell cultures of dispersed rat anterior pituitary glands

Dispersed cells from female rats were treated for 48 h with either no steroid or with E_2 (1 nM). Cells were then incubated in medium containing either no drug (baseline), ionomycin (30 μM) or PDBu (300 nM), with steroids if appropriate, and [^{3}H]-AA release was determined. The statistical significance of the effects of ionomycin and PDBu were compared to baseline [^{3}H]-AA release (*$p < 0.05$, Mann-Whitney U-test). Values are means ± SEM for the number of determinations shown in parentheses.

	[^{3}H]-AA release (% total label incorporated)	
	no steroid	**E_2**
baseline	0.60 ± 0.02 (12)	0.70 ± 0.04 (7)
ionomycin	* 0.88 ± 0.05 (8)	* 1.12 ± 0.10 (5)
PDBu	0.61 ± 0.05 (12)	* 1.22 ± 0.20 (4)

CHAPTER 6

RADIOLIGAND BINDING STUDIES WITH [^{3}H]-N,N-DIMETHYLSTAUROSPORINE AS AN APPROACH TO CHARACTERISE PHARMACOLOGICALLY DISTINCT FORMS OF PROTEIN KINASE C

6.1 INTRODUCTION

In previous chapters, H7-resistant PKC-like kinases were described, together with evidence for their role in PKC-activator induced LH and GH release and in the LHRH priming phenomenon in pro-oestrous rat anterior pituitary tissue. However, the relationship of these H7-resistant forms of PKC to the known isoforms is not clear. The known PKC isoforms have distinct tissue distributions and it may be possible to correlate this pattern with the tissue distribution of the H7-resistant form. For example the H7-sensitivity of PKCs purified from different tissue sources could be determined using a cell-free assay of PKC activity, in the same way as we examined the H7-sensitivity of anterior pituitary Ca^{2+}-independent and -dependent PKCs in Chapter 3 (Thomson, Johnson, MacEwan and Mitchell, submitted). Alternatively, since both staurosporine and H7 interfere with enzyme activity by interacting with, or near to, the ATP binding site (Nakadate *et al,* 1988), it may be possible to determine the tissue distribution of the H7-resistant PKC by H7 displacement of binding of the radiolabelled derivative of staurosporine, [^{3}H]-N, N-dimethylstaurosporine ([^{3}H]-DMS) to PKC. In this chapter, experiments are described in which radioligand binding studies, using [^{3}H]-DMS, were used in an attempt to characterise PKCs with respect to any differential pharmacology of PKC inhibitors.

6.2 SPECIFIC METHODOLOGY

Equilibrium displacement of [^{3}H]-DMS binding by PKC inhibitors to sites in different tissues was carried out as described in Chapter 2, section 2.2.7. Male rat lung or midbrain was homogenised in 5 vol of homogenisation buffer, and cytosol was prepared as described in section 2.2.7. Staurosporine and Ro 31-8220 were made up as stock solutions at 10^{-3} M in DMF and H7 was made up as a stock solution at 10^{-3} M in distilled H_2O. Further dilutions were carried out in assay buffer. Total binding (TB) was measured in the absence of unlabelled PKC inhibitor and non-

specific binding (NSB, which was approximately 25% of TB) was determined in the presence of 3 μM staurosporine.

6.3 RESULTS

Figure 6.1 shows the displacement by staurosporine of [^{3}H]-DMS binding to specific recognition sites in cytosolic preparations from male rat lung and midbrain. These tissues were chosen because rat midbrain contains mRNA for a wide range of different PKC isoforms (Scott-Young III, 1989) and lung, like anterior pituitary, contains a PKC activity which is unusually resistant to H7 (MacEwan, Johnson, Ison, Thomson and Mitchell, in preparation). Staurosporine displaced [^{3}H]-DMS binding to sites in lung and midbrain cytosol with Hill slopes of much less than one (Table 6.1) consistent with both tissues having more than one site with distinctly different affinities for staurosporine. Midbrain cytosol contained sites sensitive to particularly low concentrations of staurosporine (with 27 ± 4% displacement at just 0.02 nM) that were apparently absent in lung. At concentrations of staurosporine of 200 nM and above, a further phase of displacement of binding was observed in both midbrain and lung; the specificity of this component is not clear.

The staurosporine derivative, Ro 31-8220, which shows greater selectivity for PKC than staurosporine, also displaced [^{3}H]-DMS binding to sites in male rat lung and midbrain cytosol with a Hill number of less than 1 (Figure 6.2 and Table 6.2) suggesting that Ro 31-8220 can bind to several sites within these tissues with different affinities. Although displacement studies using Ro 31-8220 did not reveal any sites in midbrain cytosol which can obviously recognise Ro 31-8220 with particularly high affinity, Ro 31-8220 displayed slightly greater potency at sites in midbrain than in lung (Table 6.2).

Unlike staurosporine and Ro 31-8220, H7 displaced [^{3}H]-DMS binding to sites in both lung and midbrain cytosol with similar potencies (Figure 6.3). The potency of H7 in comparison to staurosporine was extremely low, with 500 μM H7

displacing only approximately 40% of total binding. It was clear that these drugs recognise sites on the enzyme which are not identical and that any effect of high concentrations of H7 on binding could well be attributed to completely non-specific actions, rather than allosteric modulation of the [^{3}H]-DMS binding domain. Therefore, no further studies were carried out.

6.4 DISCUSSION

The purpose of these [^{3}H]-DMS binding studies was to characterise the H7-resistant PKC-like kinase, described in earlier experiments to be involved in LHRH priming, PKC activator-induced LH and GH release and in the control of PLA_2 activity in the anterior pituitary, by correlating its tissue distribution with that of the known PKC isoforms. However, H7 only weakly displaced [^{3}H]-DMS binding to sites in both lung and midbrain cytosol, supporting other data (Gross *et al,* 1990; Herbert *et al,* 1990b), and was unable to distinguish sites unique to either tissue. As a consequence, under the present experimental conditions, H7 is unlikely to have any allosteric modulatory effects on the characteristics of [^{3}H]-DMS binding, except at high, possibly non-specific concentrations. Since these results indicate that the recognition sites for staurosporine and H7 on the protein are clearly not identical it was not possible to use this technique to characterise the distribution of the H7-resistant PKC(s). Interestingly, other workers have observed that [^{3}H]-DMS binding to purified PKC is enhanced in the presence of Mg^{2+} and histone substrate, but not Ca^{2+} or phosphatidylserine (Gross *et al,* 1990). It is possible that the characteristics of H7 binding to PKC may also be altered in the presence of factors involved in enzyme catalysis, and it will be of interest to determine whether these factors can also modulate the ability of H7 to displace [^{3}H]-DMS binding.

The observation that H7 is unable to displace [^{3}H]-DMS binding is of interest since, although both staurosporine and H7 are suggested to inhibit enzyme activity by acting close to the ATP-binding site of the protein, these results would

suggested that different binding sites for each inhibitor exist on the catalytic domain of the kinase. Consistent with this hypothesis, H7, but not staurosporine is suggested to be a competitive inhibitor with respect to ATP. The concentrations of staurosporine required to inhibit PKC-mediated cellular responses are much lower than would have been predicted for competitive inhibition with ATP (Rüegg and Burgess, 1989). However, although H7 has been suggested to act in a kinetically competitive manner with respect to ATP (Hidaka *et al,* 1984), it is not entirely clear whether the H7 recognition site is completely concurrent with that of ATP, since H7 can only provide partial protection against denaturation of the ATP site by covalent chemical reagents (Ohta *et al,* 1988).

These binding studies have also shown that lung and midbrain cytosol have more than one binding site for staurosporine and Ro 31-8220 with different affinities (Figure 6.1 and Table 6.1). Binding to midbrain sites was more sensitive to displacement by staurosporine than binding to lung sites suggesting that midbrain, but not lung, expresses a kinase (or group of kinases) which is highly sensitive to staurosporine; much more so than has been previously reported for staurosporine on purified PKC activity (Chapter 3, Gross *et al,* 1990). A further phase of displacement of [^{3}H]-DMS binding by staurosporine occurring at concentrations of 200 nM and above suggested that there may be a minor population of specific binding sites (~15%), present in both midbrain and lung, that are particularly insensitive to staurosporine. Since staurosporine has relatively little selectivity for PKC and can inhibit the activity of other kinases (see Rüegg and Burgess, 1989 for review), it is likely that these multiple recognition sites for staurosporine may represent binding to one or more of a number of kinases. It is not yet clear which of these binding site components represent ligand bound to PKC, although both [^{3}H]-DMS binding to purified preparations of PKC and the enzymatic activity of purified PKC are inhibited by staurosporine over a concentration range of 1 - 5 nM (Tamaoki *et al,* 1986; Gross *et al,* 1990; Herbert *et al,* 1990b). However, staurosporine can inhibit the activity of

other protein kinases with IC_{50} values in the same range as the inhibition of PKC activity. For example, staurosporine has been reported to inhibit PKA activity with an IC_{50} of 8 nM, Ca^{2+}/calmodulin-dependent protein kinases with an IC_{50} of 10 - 40 nM, $pp^{60v\text{-}src}$ and pp^{70trk} tyrosine kinases with IC_{50} values over a range of 3 - 10 nM, PKG with an IC_{50} of 8.5 nM, and myosin light chain kinase with an IC_{50} of 1.3 nM (Tamaoki *et al,* 1986; Davis *et al,* 1989; Rüegg and Burgess, 1989; Herbert *et al,* 1990b; Hidaka and Kobayashi, 1992; Ohmichi *et al,* 1992). Furthermore, staurosporine displaces [^{3}H]-DMS binding to purified PKA and Ca^{2+}/calmodulin-dependent protein kinase with IC_{50} values of 1 and 5 nM respectively (Herbert *et al,* 1990b). It would seem, therefore, that staurosporine displacement of [^{3}H]-DMS binding over a concentration range of 1 - 40 nM may represent interactions with a number of different kinases, including PKC. In addition, the high and low affinity sites for staurosporine that were detected in rat midbrain do not overtly appear to represent binding to PKC, or any of the other kinases described above. Interestingly, staurosporine is reported to inhibit cell proliferation with an IC_{50} value of 4 pM, which is much lower than the IC_{50} of 3 nM quoted for the inhibition of PKC activity (Tamaoki *et al,* 1986). Although the identity of the staurosporine-sensitive kinase involved in cell proliferation is unknown, this enzyme may be a member of the growth factor-associated tyrosine kinases that are involved in cell division. The identity of the rat brain kinase which displays high affinity for staurosporine is also unclear, but it may be related to the enzyme involved in cell proliferation. A number of kinases have been described which are inhibited by staurosporine with lower potency. For example, mitogen-activated protein kinase (MAP kinase) activity from rat hippocampus is inhibited by staurosporine with an IC_{50} value of 1.5 ± 1.2 μM (Leslie, Johnson and Mitchell, unpublished observations). Furthermore, staurosporine, at a concentration as high as 1 μM, is unable to inhibit the epidermal growth factor receptor-associated tyrosine kinase (Ohmichi *et al,* 1992). Thus, the low affinity [^{3}H]-DMS binding sites may represent binding to either certain growth

factor-receptor tyrosine kinases, a member of the MAP kinase family or other unspecified kinase enzymes, perhaps including novel species.

Since it is clear that [^{3}H]-DMS binds to protein kinase, other than PKC, we attempted to distinguish binding to PKC from binding to other kinases by displacement studies using the selective PKC inhibitor, Ro 31-8220. Although Ro 31-8220 is reported to inhibit PKC action with 100-fold greater selectivity than staurosporine (Davis *et al,* 1989), these studies did not reveal any binding sites with obvious selectivity for Ro 31-8220. It is likely that the relative potencies of Ro 31-8220 on various types of protein kinase are not sufficiently different for this inhibitor to clearly distinguish between various different kinases in the displacement of [^{3}H]-DMS binding, and perhaps ligand binding to authentic PKC may represent a very small fraction of the sites labelled here. Recently developed staurosporine analogues, such as Ro 31-8425, show 350-fold greater selectivity for PKC (Nixon *et al,* 1992) and may prove to be a more useful tool to pursue the approach attempted here. Ideally, the use of radiolabelled PKC-selective analogues of staurosporine (such as the Ro compounds) would greatly simplify the analysis of specific radioligand binding to PKC. However, until these selective radioligands are developed, it may be possible to dissociate [^{3}H]-DMS binding to PKC from binding to other kinases by a number of different approaches. For example, phorbol ester treatment of the tissue, prior to homogenisation, will cause PKC (at least the A series isoforms) to translocate to the membrane, depleting the cytosol of PKC. [^{3}H]-Dimethylstaurosporine binding studies to phorbol ester-treated cytosol will provide a profile which represents binding more predominantly to kinases other than PKC. However, this approach has a number of drawbacks since it is clear that the ζ and λ PKC isoforms do not respond overtly to phorbol esters (Ono *et al,* 1989; Y. Nishizuka, personal communication). In addition, these studies are clearly limited since they will not allow further study of selective binding to PKC itself. Alternatively, it may be possible to examine selective [^{3}H]-DMS binding to PKC by preparing samples of purified enzyme. For example,

PKC activity can be partially purified from cell homogenates by passage through DEAE cellulose (as was described for the PKC activity assays in Chapter 3) and this can be further resolved into different peaks of enzymatic activity by hydroxyapatite column chromatography (Kikkawa *et al,* 1987). Possible contamination of the purified PKC preparation by other kinases could then be assessed by comparing the number of [^{3}H]-DMS binding sites in the preparation to the number of [^{3}H]-PDBu binding sites. Alternatively, preparations of specific purified PKC isoforms can be obtained by expressing the cDNA for the appropriate isoform in a suitable system, for example, the baculovirus expression system, following which, the enzyme can be purified. It will be of interest to see whether the profile of [^{3}H]-DMS binding and displacement varies between different isoforms of PKC.

Although these experiments did not provide an answer as to the identity of the H7-resistant PKC form that has a function in gonadotroph and somatotroph responses, [^{3}H]-DMS binding studies may be useful in other investigations. For example, the site of action on PKC of new pharmacological tools may be determined by comparing the displacement of [^{3}H]-DMS binding to the catalytic domain of the enzyme with displacement of [^{3}H]-PDBu binding to the regulatory domain. It should be said, however, that this approach was not successful when considering the site of action of H7 which, like staurosporine, is though to act on the catalytic domain, in close proximity to the ATP recognition site (Nakadate *et al,* 1988). In equivalent experiments, displacement of [^{3}H]-PDBu binding by PKC activators such as DOG, which can mimic some actions of phorbol esters but not others (Chapters 3 and 5), may provide an insight into the identity of PKCs which differ in their PKC activator pharmacology (MacEwan *et al,* 1992a).

Since these [^{3}H]-DMS binding studies were carried out, the distribution of the H7-resistant PKC has been determined by an alternative means (D. MacEwan, PhD Thesis, University of Edinburgh). In these studies, the effect of H7 was examined on PDBu-induced, phosphatidylserine-dependent, histone IIIS kinase

activity that was partially-purified from different tissue sources. Of the tissues examined, male and female rat anterior pituitary and rat lung cytosol, but not male rat frontal cerebral cortex, cerebellum, spleen, midbrain or COS 7 cells, contained Ca^{2+}-independent, PDBu-induced, H7-resistant kinase activity. This tissue distribution does not correlate with the distribution of any of the characterised isoforms of PKC. Since the H7-resistant PKC form detected in these cell-free assays does not require Ca^{2+} for activation, it would seem that this enzyme may represent a member of the B series isoforms. However, histone is reported to be a poor substrate for the B series PKC isoforms (Ohno *et al,* 1988; Schaap *et al,* 1989; Schaap and Parker, 1990; Olivier and Parker, 1991) thus the Ca^{2+}-independent, H7-resistant histone kinase activity in anterior pituitary and lung cytosol does not correlate well with the activity of a B series isoform. Furthermore, it is clear that the Ca^{2+} dependency of the A series isoforms may change under certain assay conditions (Wolf *et al,* 1984; Bazzi and Nelsestuen, 1987) and according to the phosphorylation state of the enzyme (Pelech *et al,* 1991), suggesting that the H7-resistant form could be a modified state of an A series isoform. Using purified recombinant preparations, the δ and ε PKC isoforms have been shown to be much more sensitive to H7 than the Ca^{2+}-independent kinase activity detected in lung and anterior pituitary (Schaap and Parker, 1990; Uchida *et al,* 1991). The H7-resistant PKC here was also readily activated by PDBu, suggesting that the phorbol ester-insensitive ζ and λ PKC isoforms (Ono *et al,* 1989; Y. Nishizuka, personal communication) do not represent the H7-resistant PKC. It would seem then that the H7-resistant kinase is either a poorly characterised B series PKC, such as η or θ, a modified A series isoform or is a kinase which has not been fully characterised. Further studies are required to determine the identity of this H7-resistant PKC. Experiments exploring immunodepletion of certain PKC isoforms, using specific polyclonal antipeptide antibodies and their precipitation with protein A Sepharose, followed by assessment of the H7-sensitivity of the remaining enzymes, may eliminate some of the candidates for the H7-resistant kinase.

FIGURE 6.1

Displacement of [^{3}H]-N,N-dimethylstaurosporine ([^{3}H]-DMS) binding to sites in (a) midbrain and (b) lung cytosol by staurosporine

Data were calculated as a % of total binding (TB), and the curve was fitted using the non-linear, error-weighted iterative curve fitting programme, P.fit (Biosoft, Cambridge). All values are mean ± SEM, n = 4 - 12 determinations.

Figure 6.1

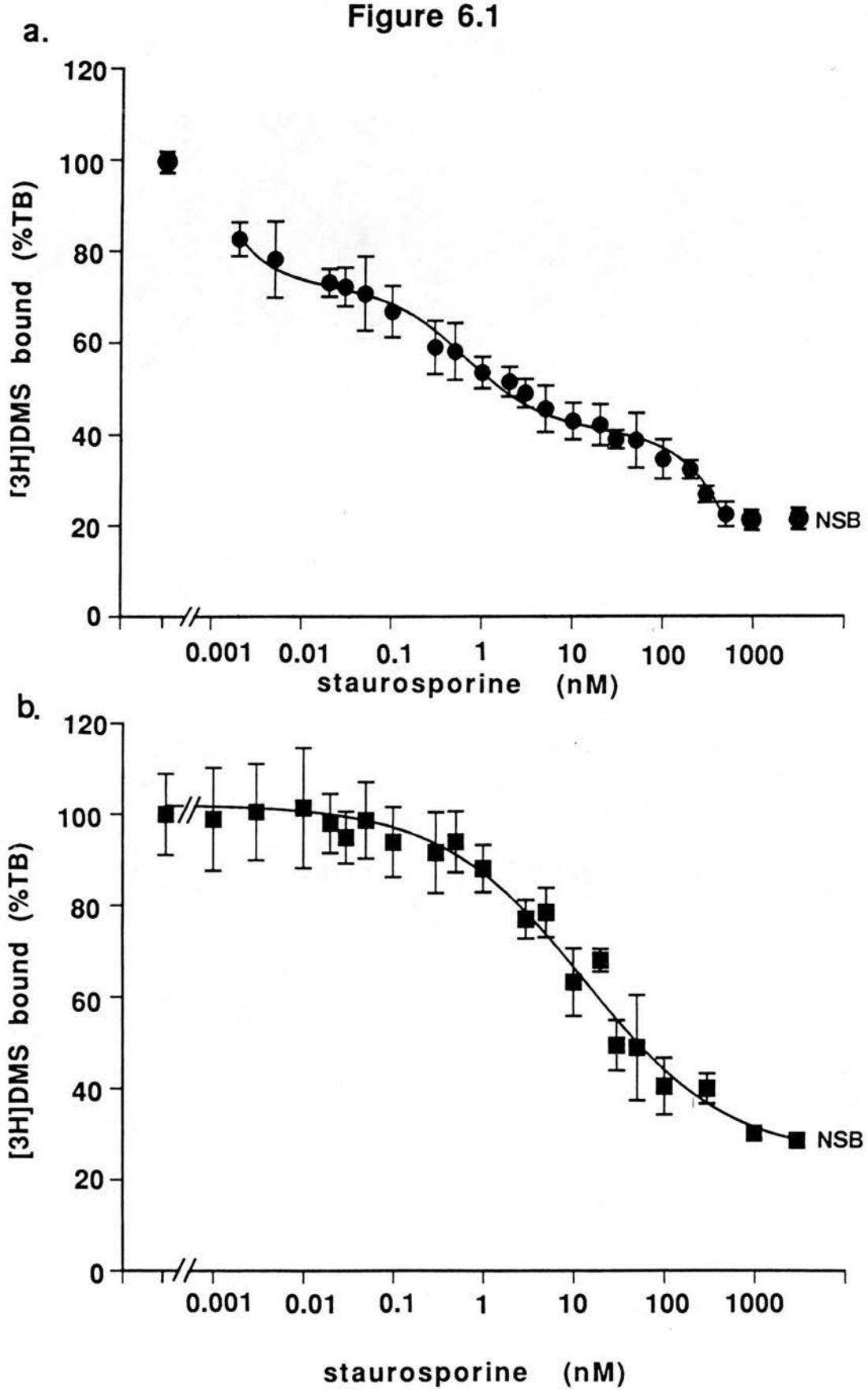

a.
120
100
80
60
40
20
0
[3H]DMS bound (%TB)
NSB
0.001
0.01
0.1
1
10
100
1000
staurosporine (nM)
b.
120
100
80
60
40
20
0
[3H]DMS bound (%TB)
NSB
0.001
0.01
0.1
1
10
100
1000
staurosporine (nM)

FIGURE 6.2

Displacement of [^{3}H]-N,N-dimethylstaurosporine ([^{3}H]-DMS) binding to sites in (a) midbrain and (b) lung cytosol by Ro 31-8220

Data were calculated as a % of total binding (TB), and the curve was fitted using the non-linear, error-weighted iterative curve fitting programme, P.fit (Biosoft, Cambridge). All values are mean ± SEM, n = 4 - 12 determinations.

Figure 6.2

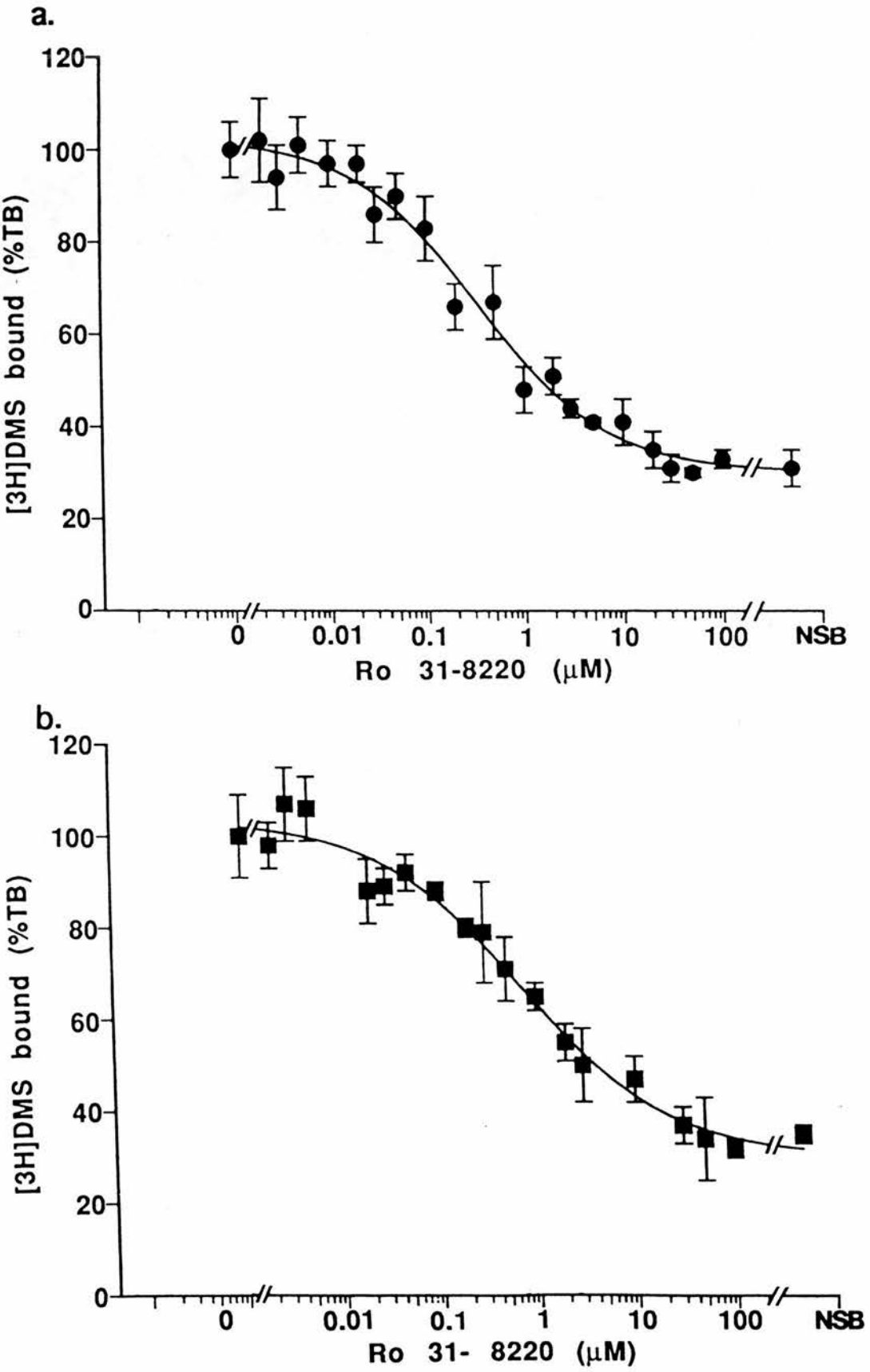

a.
120
100
80
60
40
20
0
[3H]DMS bound (%TB)
0
0.01
0.1
1
10
100
NSB
Ro 31-8220 (μM)
b.
120
100
80
60
40
20
0
[3H]DMS bound (%TB)
0
0.01
0.1
1
10
100
NSB
Ro 31- 8220 (μM)

FIGURE 6.3

Displacement of [^{3}H]-N,N-dimethylstaurosporine ([^{3}H]-DMS) binding to sites in (a) midbrain and (b) lung cytosol by H7

Data were calculated as a % of total binding (TB), and the curve was fitted using the non-linear, error-weighted iterative curve fitting programme, P.fit (Biosoft, Cambridge). All values are mean ± SEM, n = 4 - 12 determinations.

Figure 6.3

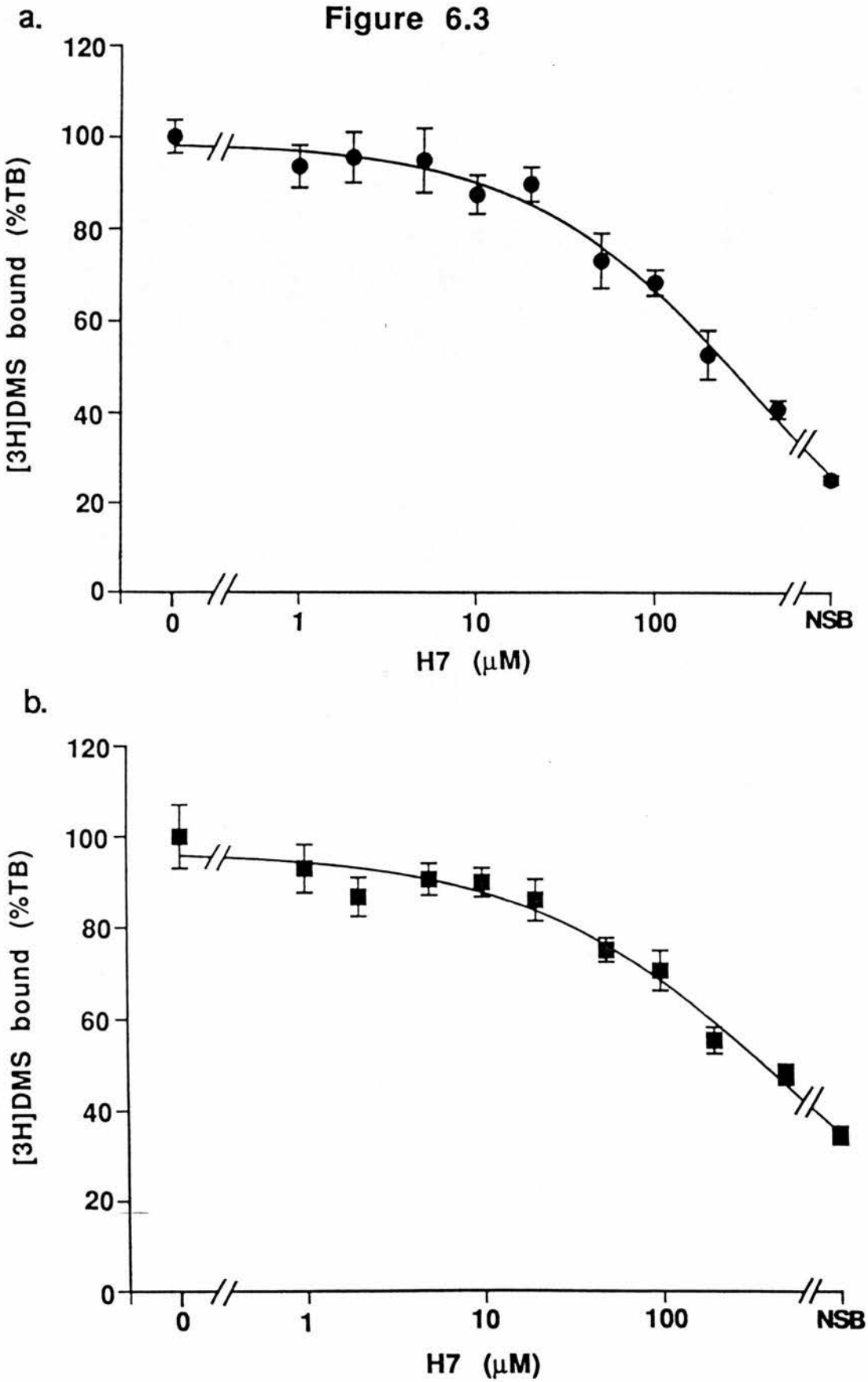

a.
120
100
80
60
40
20
0
[3H]DMS bound (%TB)
0
1
10
100
NSB
H7 (μM)
b.
120
100
80
60
40
20
0
[3H]DMS bound (%TB)
0
1
10
100
NSB
H7 (μM)

TABLE 6.1

Hill slope and IC_{50} values for staurosporine displacement of [^{3}H]-N,N-dimethylstaurosporine ([^{3}H]-DMS) binding to sites in rat lung and midbrain cytosol

Values were obtained by fitting the data using the non-linear, error-weighted iterative curve fitting programme, P.fit. The IC_{50} value was defined as being that concentration of staurosporine which displaced 50% of total binding. All values are shown as mean ± SEM or 4 - 12 determinations.

	midbrain	lung
Hill slope	0.22 ± 0.05	0.58 ± 0.07
IC_{50} (nM)	0.23 ± 0.06	9.38 ± 1.93

TABLE 6.2

Hill slope and IC_{50} values for Ro 31-8220 displacement of [^{3}H]-N,N-dimethylstaurosporine ([^{3}H]-DMS) binding to sites in rat lung and midbrain cytosol

Values were obtained by fitting the data using the non-linear, error-weighted iterative curve fitting programme, P.fit. The IC_{50} value was defined as being that concentration of Ro 31-8220 which displaced 50% of total binding. All values are shown as mean ± SEM for 4 - 12 determinations.

	midbrain	**lung**
Hill slope	0.66 ± 0.1	0.56 ± 0.1
IC_{50} (μM)	0.35 ± 0.08	0.77 ± 0.2

CHAPTER 7

OVERVIEW

OVERVIEW

The involvement of PKC in the signal transduction system of the LHRH receptor in anterior pituitary gonadotrophs has been a controversial issue for a number of years (see Conn, 1989 for review). Although LHRH can stimulate the redistribution of PKC activity from the cytosolic to the particulate fraction in anterior pituitary cell cultures (Hirota *et al,* 1985; McArdle and Conn, 1986), and PKC activators can induce LH release (Conn *et al,* 1985; Naor and Eli, 1985; Chang *et al,* 1986a, 1986b; Turgeon and Waring, 1986; Johnson and Mitchell, 1989; Chapter 3), it has been difficult to assess the involvement of PKC in LHRH action since selective inhibitors of this kinase have not been available until recently. However, we have shown here that the recently developed highly selective PKC inhibitor, Ro 31-8220, and a number of older PKC inhibitors (staurosporine, K252a and H7) could prevent the induction of LHRH priming, measured in pro-oestrous rat anterior pituitary tissue *in vitro,* without inhibiting the initial secretory response to LHRH (Chapter 3). These results are in agreement with previous observations using less selective PKC inhibitors which suggest that PKC activation is not essential for acute gonadotrophin release in response to LHRH (McArdle *et al,* 1987), but may be required, in part, for later responses to LHRH (Hirota *et al,* 1985; Chang *et al,* 1987b; Fink *et al,* 1990). In addition to having a role in LHRH priming as shown here, PKC is also suggested to mediate LHRH-induced changes in gonadotrophin gene expression and LH synthesis in cultured, dispersed anterior pituitary cells (Andrews *et al,* 1988; Stojilkovic *et al,* 1988b). However, PKC-dependent positive actions on gonadotrophin synthesis are not apparent until after 12 hours of LHRH treatment (Andrews *et al,* 1988), suggesting that this action of PKC may not be important for LH release responses measured over 2 - 3 consecutive hourly exposures to LHRH, as used in these studies. Certainly, in pro-oestrous rat hemipituitary pieces, there is no significant change in the total amount of gonadotrophin following an initial exposure to LHRH (Pickering

and Fink, 1979), suggesting that, in this model of anterior pituitary cell function, LHRH does not induce any significant change in gonadotrophin synthesis.

Most interestingly, the PKC that is involved in the induction of LHRH priming, has unusual PKC inhibitor pharmacology. That is, the LHRH priming response was readily inhibited by staurosporine and Ro 31-8220, but was relatively resistant to H7 in comparison to certain other PKC-mediated responses (Hidaka *et al,* 1984; Chapter 3). The identity of this H7-resistant PKC with respect to the known PKC isoforms is unknown, although evidence from biochemical assays of cytosolic PKC activity indicate that this PKC is a Ca^{2+}-independent type. However, of the known B series isoforms, both δ and ε PKC are more sensitive to H7 (Schaap and Parker, 1990; Uchida *et al,* 1991) than the kinase involved in priming. Furthermore, the ζ and λ PKC isoforms, unlike the H7-resistant kinase in rat anterior pituitary tissue, are not responsive to phorbol esters (Ono *et al,* 1989; Y. Nishizuka, personal communication). Thus, it would appear that a poorly characterised B series PKC isoform, such as η or θ PKC or some unknown PKC-like enzyme may represent the PKC involved in LHRH priming. However, histone has been reported to be a poor substrate for the characterised Ca^{2+}-independent PKC forms (Ohno *et al,* 1988; Huang, 1989; Schaap and Parker, 1990), suggesting that the H7-resistant, Ca^{2+}-independent histone kinase activity detected in anterior pituitary cytosol may not result from the activation of a B series isoform. It is now clear that the Ca^{2+}-dependency of the A series PKC isoforms is not as clear-cut as might have been perceived at first. Autophosphorylation of the β PKC isoform can reduce its requirement for Ca^{2+} (Pelech *et al,* 1991) and the Ca^{2+}-dependency of the A series isoforms alters under different assay conditions (Wolf *et al,* 1984; Bazzi and Nelsentuen, 1987). It is possible, therefore, that the priming kinase may represent a modified A series PKC isoform.

In an attempt to further characterise this novel PKC-like kinase, H7 displacement of [^{3}H]-DMS binding to cytosolic sites from different tissues sources

was examined (Chapter 6) but was found to be of limited use since the binding sites for staurosporine and H7 were clearly not identical. Nevertheless, this observation is of interest since the difference in the potency of H7, but not staurosporine, on Ca^{2+}-independent compared with Ca^{2+}-dependent PDBu-induced cytosolic PKC activity in anterior pituitary (Chapter 3) may be accounted for by the fact that each inhibitor binds to slightly different sites on the enzyme. It is clear that further studies are required to identify the H7-resistant PKC involved in LHRH priming. One approach may be to partially purify anterior pituitary PKC and immunodeplete certain PKC isoforms using specific polyclonal antipeptide antibodies and protein A Sepharose. The H7-sensitivity of the remaining enzymes could then be assessed, and this may eliminate some of the candidates for the H7-resistant kinase. Following separation and purification of the H7-resistant PKC, fragments of the protein may be microsequenced, and oligonucleotides synthesised which could be used to probe a rat anterior pituitary cDNA library to identify full length clones encoding this kinase. Comparing even fragments of amino acid sequence or the nucleotide sequence of this clone with those of the known PKC isoforms may clarify the identity of the kinase involved in LHRH priming. However, problems may arise using this approach to identify the priming kinase. For example, the pituitary consists of a heterogeneous cell population, and cell types, other than gonadotrophs, may express H7-resistant PKC-like kinases which function in processes other than LHRH priming. Indeed, an H7-resistant form of PKC has been implicated in the dual control of anterior pituitary L-type Ca^{2+} channel activity (Mitchell *et al,* 1990), and it is possible that this represents a kinase which is distinct from that involved in LHRH priming. Using a clonal cell line, such as the α T3-1 gonadotrophs, to purify and characterise the priming kinase would, at first sight, seem to overcome the problem of contamination from PKCs from other cell types. However, it is clear that the role of PKC in LHRH responses in α T3-1 cells differs from that in rat anterior pituitary pieces (Chapter 4), suggesting that this cell line may be of limited use in the identification of the priming

kinase. However, until additional studies are carried out, it is uncertain whether the H7-resistant form of PKC, that is involved in LHRH priming, represents one of the known isoforms of PKC or if it is a novel, as of yet, unidentified form of PKC or a closely related kinase.

Preliminary evidence from other studies has suggested that PLA_2 activation may be required for secretory responses to LHRH in cultures of dispersed anterior pituitary cells (Naor and Catt, 1981; Chang *et al,* 1986a, 1987b), leading us to investigate a possible role of PLA_2 in LHRH responses in pro-oestrous rat anterior pituitary tissue (Chapter 4). Indeed, it appears that the induction of LHRH priming is dependent upon the activation of PLA_2 since LHRH priming, but not initial LHRH-induced gonadotrophin release was blocked by a number of PLA_2 inhibitors and LHRH could induce an increase in [^{3}H]-AA release from pre-labelled pro-oestrous rat hemipituitary pieces, a response which was blocked by inhibitors of PLA_2, but not of DAG lipase activity. In addition, LHRH-induced modulation of PLA_2 activity apparently occurs by a route which is dependent upon both protein synthesis and the actions of an H7-resistant form of PKC. One possibility is that the H7-resistant PKC, which induces LHRH priming, controls the synthesis of a protein which can activate phospholipase A_2. Certainly, preliminary results from Northern blot analysis have shown that LHRH treatment of pro-oestrous rat anterior pituitary tissue can increase PLAP mRNA levels (Lutz and Mitchell, unpublished observations), implying that this protein may, in some way, be involved in LHRH receptor responses. However, additional experiments are required to determine the physiological relevance of PLAP in LHRH receptor signalling. Biologically-active peptide fragments of phospholipase A_2-activating protein are available, and it would be of interest to see what affect these have on gonadotroph PLA_2 activity and gonadotroph responsiveness. Monoclonal antibodies against PLAP and PLAP antisense oligonucleotides may, in principle, also be useful since one could attempt to inhibit LHRH priming using these tools. However, such studies require dispersed, permeabilised cells which, unfortunately, do

not response to LHRH in the same manner as intact tissue (see section 1.?.3 for discussion). It is, of course, possible that during LHRH receptor activation, a protein, other than PLAP, is synthesised which may induce LHRH priming. However, the identity of any such a protein is uncertain.

The results presented in this thesis have led us to postulate a simplified model of the intracellular changes that may occur following LHRH stimulation of the pro-oestrous rat gonadotroph, leading to gonadotrophin release and priming (Figure 7.1). Simulation of the LHRH receptor causes a G-protein-dependent activation of PLC, inositol phospholipid breakdown and production of $Ins(1,4,5)P_3$ and DAG. In addition, since LHRH treatment of α T3-1 cells can activate PLD and thereby increase PtdOH levels (Netiv *et al,* 1991), it is possible (by the subsequent action of phosphatidate phosphohydrolase) that LHRH can also increase gonadotroph DAG levels independently of $Ins(1,4,5)P_3$ production. Inositol 1,4,5-trisphosphate will mobilise Ca^{2+} from intracellular stores (Berridge and Irvine, 1984, 1989) which, together with Ca^{2+} influx through plasma membrane channels, will contribute to early secretory responses to LHRH, perhaps by affecting Ca^{2+}/calmodulin-dependent processes (Conn *et al,* 1981a). In parallel, DAG will activate the H7-resistant PKC which is involved in the priming response. It is possible that the origin of this DAG and its side chain composition might confer selectivity in activating particular PKCs. By inducing the synthesis of a protein (perhaps PLAP), the H7-resistant PKC enhances PLA_2 activity and AA release. Arachidonic acid may mediate priming by having an action on various aspects of cell function, but by a mechanism which is perhaps not dependent upon metabolism of the fatty acid. For example, AA has been reported to activate PLC (Irvine *et al,* 1979), possibly by increasing intracellular Ca^{2+} levels which may alter the activity of this enzyme. Such an effect of AA on gonadotroph PLC activity may account for the facilitated production of $Ins(1,4,5)P_3$ and increased Ca^{2+} mobilisation response that is associated with priming (Mitchell *et al,* 1988). Arachidonic acid can cause Ca^{2+} mobilisation from intracellular stores,

including those which are sensitive to Ins(1,4,5)P_3 (Beaumier *et al,* 1987), can activate certain isoforms of PKC (Naor *et al,* 1988; Shearman, 1989a), and can modulate the activity of certain types of ion channel. Any combination of these cellular actions of AA may play an important role in LHRH priming. However, since PKC inhibitors were unable to prevent LHRH-induced LH release from tissue which had been previously primed, it is unlikely that the expression of priming is mediated by an action of AA on PKC activity. Nevertheless, it is possible that AA may activate other kinases involved in the induction of LHRH priming. Furthermore, LHRH priming does not require extracellular Ca^{2+} (Pickering and Fink, 1979), is unaffected by thapsigargin (a depletor of intracellular Ca^{2+}) (Thomson, Mitchell and Johnson, unpublished observations) and is unaffected by calmodulin antagonists or Ca^{2+}/calmodulin-dependent kinase inhibitors (Mitchell, Wolbers, Johnson and Thomson, unpublished observations). Thus, changes in intracellular Ca^{2+} levels, brought about by AA, AA metabolites, or by some other means may not be important for the phenomenon of LHRH priming.

In addition to increasing cellular levels of free AA, PLA_2 action will also liberate lysophospholipid, which can be metabolised to PAF. Platelet-activating factor can bind to its own membrane associated receptors and induce inositol phospholipid turnover (Shulka, 1991). Northern blot analysis has shown that a small but significant amount of mRNA for PAF receptor is present in the dog pituitary (Bito *et al,* 1992), suggesting that PAF receptor may be present on some pituitary cells. The possibility that PAF may have an autocrine or paracrine action in the anterior pituitary which may contribute to the LHRH priming effect is an interesting prospect.

Although the early secretory response to LHRH is dependent upon Ca^{2+} mobilisation from both intracellular and extracellular pools (Davidson *et al,* 1991), LHRH priming can occur in Ca^{2+}-free medium (as assessed by the ratio of LH release measured in the 2nd h of LHRH incubation to the 1st h incubation) (Pickering and Fink, 1979) and is unaffected by thapsigargin (Thomson, Mitchell and Johnson,

unpublished observations). Furthermore, the H7-resistant, PKC-like kinase found in anterior pituitary cytosol is active at extremely low (< 3 nM) Ca^{2+} levels, and its activity was not obviously promoted by raising Ca^{2+} levels (Chapter 3). These observations suggest that the induction of LHRH priming may be a Ca^{2+}-independent process. However, although increased levels of intracellular Ca^{2+} may not be a pre-requisite for the amplification of gonadotroph responsiveness, PKC action may enhance the LHRH secretory response by reducing the Ca^{2+} requirement of the Ca^{2+}-dependent process of hormone release. Indeed, the Ca^{2+} levels measured in gonadotrophs after chronic LHRH treatment (Limor *et al,* 1987) may be lower than those levels that are sufficient alone to trigger secretion (Rink *et al,* 1982). Thus, a possible synergy between Ca^{2+} and PKC may be important for low levels of Ca^{2+} to be functionally significant. However, in pro-oestrous rat hemipituitaries, it was clear that a PKC-independent release of gonadotrophin can still occur during a 2nd h exposure to LHRH (Chapter 3). Thus, the actions of cellular processes, other than PKC, perhaps Ca^{2+}-dependent events, are sufficient alone to induce at least part of the later phase of LHRH-induced LH release in this model. Nevertheless, Ca^{2+} ionophores and PKC activators can synergise to release levels of LH from dispersed cells in culture that are greater than those measured in the presence of either secretagogue alone (Harris *et al,* 1985; Naor and Eli, 1985), indicating that PKC may serve to amplify hormone release induced by other cellular factors. In pro-oestrous rat tissue, it is possible, therefore, that the actions of the 'priming kinase' may amplify gonadotrophin release induced by the facilitated intracellular Ca^{2+} mobilising response which is associated with LHRH priming (Mitchell *et al,* 1988). Although the mechanism by which the actions of PKC and other factors, like Ca^{2+} may interact to facilitate gonadotrophin release is unknown, studies using GH_4C_1 anterior pituitary cells may provide a clue as to how this interaction occurs. During TRH stimulation of GH_4C_1 cells, PKC action is important for the second phase of a biphasic prolactin release response, a response which requires PKC phosphorylation of and subsequent

re-organisation of the actin cytoskeleton (Kiley *et al,* 1992). During LHRH stimulation of the gonadotroph, an equivalent action of PKC on the gonadotroph cytoskeleton may account for earlier observations which have shown that priming is dependent upon microfilament integrity and is associated with movement of the secretory granules to the plasma membrane (Pickering and Fink, 1979; Fink, 1988). This action of PKC on the gonadotroph cytoskeleton may render the secretory granules more susceptible to release in response to other factors such as Ca^{2+}.

The magnitude of the LHRH priming response is dependent upon the stage of the rat oestrous cycle and it is, therefore, not surprising that the actions, and probably the expression of the H7-resistant PKC which induces priming was enhanced by E_2 treatment (Chapter 6). Although anterior pituitary tissue from ovariectomised rats did not display a visible LHRH priming response when measured *in vitro*, preliminary experiments suggested that, following E_2 replacement of ovariectomised animals, the LHRH priming response was reinstated and that this effect of E_2 may be due to an action on the expression of the H7-resistant PKC. Dispersed anterior pituitary cells, that have been maintained in E_2-free culture conditions over several days, do not exhibit LHRH priming (Speight and Fink, 1981; Chang *et al,* 1987b), which may be explained by the experiments described in Chapter 5 which show that the activity and amount of the PKC(s) involved in LH responses to PKC activators and LHRH is reduced in an E_2-free environment. It is, therefore, perhaps not surprising that some reports have concluded that PKC activation is not required for secretory responses to LHRH measured in dispersed anterior pituitary cells which have been maintained in relatively prolonged culture.

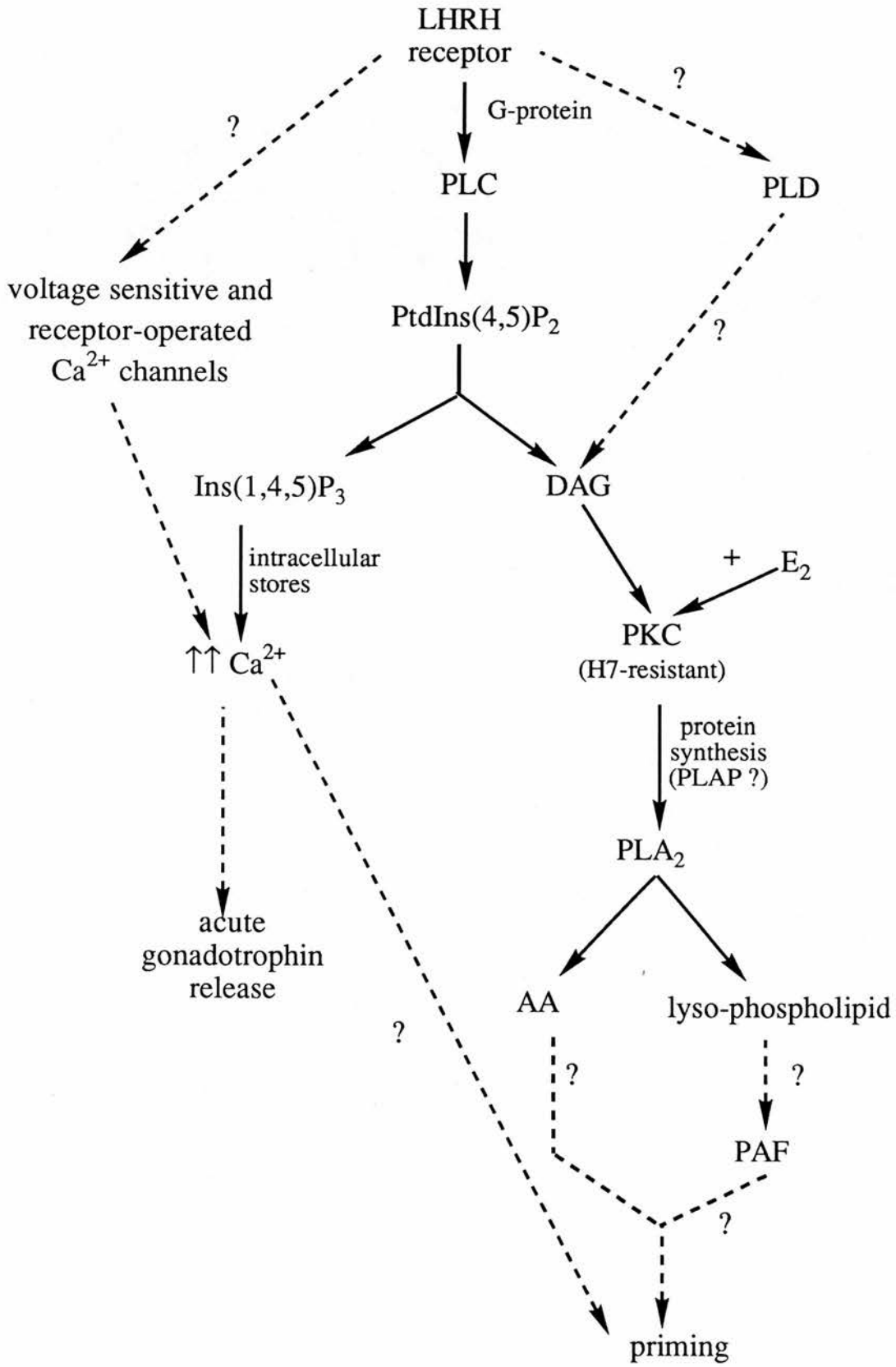
In addition to the H7-resistant PKC-like kinases involved in LHRH priming, a number of other distinct PKCs forms were found to be involved in the process of PKC activator-induced LH and GH release. These PKCs differed in their PKC inhibitor and activator pharmacology (Chapter 3). Of some interest, we found that certain PKC activators could induce LH release by a process which involved H7-

sensitive PKCs in addition to H7-resistant PKCs that were, presumably, involved in the LHRH receptor signal transduction mechanisms. Thus, following LHRH receptor stimulation, selective activation of only certain PKC forms which can modulate LH release occurs. It is possible that selective activation of one or more PKC forms by an agonist may be controlled by the particular species of DAG released following receptor stimulation, in combination with other factors such as free fatty acids and changes in intracellular Ca^{2+} concentrations or by the relative abundance of different isoforms or by compartmentalisation of certain isoforms. The difference in the PKC inhibitor and activator pharmacology of the LH and GH secretory response is of great interest since it would add further weight to evidence that the development of agents which selectively inhibit or activate certain PKC isoforms may be possible. Drugs which selectively intervene with PKC isoforms that are specifically involved in particular disease states may be novel and powerful therapeutic agents.

FIGURE 7.1

A summary of the LHRH receptor signal transduction mechanisms in the pro-oestrous rat anterior pituitary gonadotroph

Figure 7.1


LHRH
receptor
?
G-protein
?
PLC
PLD
voltage sensitive and
receptor-operated
Ca^{2+} channels
$PtdIns(4,5)P_2$
?
$Ins(1,4,5)P_3$
DAG
intracellular
stores
+
E_2
PKC
(H7-resistant)
↑↑ Ca^{2+}
protein
synthesis
(PLAP ?)
PLA_2
acute
gonadotrophin
release
AA
lyso-phospholipid
?
?
?
PAF
?
priming

APPENDIX I PREPARATION OF TISSUE CULTURE MEDIA

I.1 Preparation of stripped foetal calf serum

(i) Removal of thyroid hormones

Dowex 1 x 8 - 400 resin (chloride form) was washed extensively in distilled water, then passed through a 0.22 μm filter. The resin was then incubated with heat-inactivated foetal calf serum (100 mg wet wt/ml serum) for 5 hours on a rotor. The resin was removed by centrifugation (1000 g, 10 min). Fresh resin was added to the supernatant (100 mg wet wt/ml serum) the incubated for 15 - 18 h at room temperature. The resin was removed by centrifugation (1000 g, 10 min) followed by passing the supernatant through a 0.8 μm filter.

(ii) Removal of steroids

Activated charcoal was mixed with distilled H_2O and centrifuged (3000 g) for 20 min. The water was discarded and the charcoal was incubated with the thyroid hormone-stripped foetal calf serum (0.02 g wet wt/ml serum), initially at 37°C for 40 min followed by 30 min at 55°C. The charcoal was removed by centrifugation (10 min, 300 g) followed by passage through filters of 0.8 μm, 0.45 μm and 0.22 μm in succession.

I.2 Preparation of steroid-free DMEM

Powdered DMEM (Sigma) with 1000 mg/ml glucose and l-glutamine, but without phenol red was made as a solution, as per the manufacturers instructions, and was adjusted to pH 7.1. Arachidonic acid, docosahexaenoic acid were added as stock solutions in BSA to give final concentrations of 1 mg/l, 0.5 mg/l and 25 mg/l respectively. Penicillin and streptomycin were added to give a final concentration of 100 units/l and 0.1 mg/ml respectively. Stripped foetal calf serum was added to give a 10% final concentration. The medium was then sterilised by passing through a 0.22 μm filter.

APPENDIX II RADIOIMMUNOASSAY FOR LH, FSH AND GH

II.1 Stock solutions

(i) 0.01 M phosphate buffered saline (PBS)

8.17g NaCl

0.1g Na merthiolate

0.25g $NaH_2PO_4.2H_2O$

1.193g Na_2HPO_4 anhydrous

per litre distilled H_2O, pH 7.5

(ii) antiserum buffer

500 ml 0.01 PBS

9.306g ethylenediamine tetra-acetic acid disodium salt

2.5 ml normal rabbit serum for LH, FSH or 1.66 ml normal human serum for GH

pH to 7.0 with sodium hydroxide pellets

(iii) assay buffer (0.01 M PBS/1 % BSA)

1 litre 0.01M PBS

10 g bovine serum albumin

(iv) 0.5 M phosphate buffer

12.5 g $NaH_2PO_4.2H_2O$

59.7 g Na_2HPO_4 anhydrous

per litre distilled H_2O, pH 7.5

II.2 Iodination

Columns:	LH	15 x 1 cm column Sephadex G50 fine coated with 1.5 ml egg white (5% in PBS). Eluted with 0.01 M PBS.
	FSH	15 x 1 cm column Sephadex G50 fine coated with 1.5 ml ovalbumin (5% in PBS). Eluted with 0.01 M PBS.

	GH	50 x 1 cm Sephadex G75 fine coated with 900 µl human albumin (4.5% in PBS). Eluted with 0.05M sodium barbitone buffer (pH 8.6).
Hormones:	LH	10 µl aliquots of 250 µg/ml in 0.01M PBS.
	FSH	10 µl aliquots of 100 µg/ml in 0.01 M PBS.
	GH	10 µl aliquots of 250 µg/ml in 0.01 $NaHCO_3$.
$Na^{125}I$:		0.5 mCi in 5 µl for each iodination. (Specific activity, approximately 14 mCi/µg).
Chloramine T:	LH	2.5 mg/ml in 0.01 M PBS. 10 µl used.
	FSH	2.5 mg/ml in 0.01 M PBS. 10 µl used.
	GH	0.5 mg/ml in H_2O. 10 µl used.
Sodium metabisulphate:	LH	2.5 mg/ml in 0.01 M PBS. 25 µl used.
	FSH	2.5 mg/ml in 0.01 M PBS. 25 µl used.
	GH	1.2 mg/ml in H_2O. 10 µl used.

Method

(i) Add 0.5 M phosphate (pH 7.5) to hormone

LH: 10 µl

FSH: 15 µl

GH: 25 µl

(ii) Add 0.5 mCi $Na^{125}I$

(iii) Add chloramine T, mix gently

(iv) Reaction times: LH 1 min

FSH 45 sec

GH 25 sec

(v) Add $Na_2S_2O_5$, mix gently

(vi) Transfer to column

(vii) Elute with 0.01 M PBS

(viii) Collect fractions: 0.9 ml into 0.1 ml PBS/5% BSA for GH. 0.5 ml into 0.5 ml 0.01 M PBS/5% ovalbumin for LH and FSH.

Count radioactivity per fraction. Retain peaks protein fractions.

In all cases, iodinated hormone eluted before free iodine.

II.3 Standards

LH: NIH-LH-SI8 (0.25, 0.5, 0.75, 1.2, 2, 5, 8, 16 ng/ml in 0.01 M PBS/1% BSA) or NIDDK-rat LH-RP-2 (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 ng/ml in 0.01 M PBS/1% BSA).

FSH: NIDDK rat FSH-RP-2 (1.25, 2.5, 5.0, 10, 20, 40 ng/ml in 0.01 M PBS/1% BSA).

GH: NIDDK rat-GH-RP-2, (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 ng/ml in 0.01 M PBS/1% BSA).

II.4 Antisera

LH: NIDDK-r-LH-S-9 or NIDDK r-LH-S-10, $^{1}/_{45,000}$ dilution (stock).

FSH: NIDDK r-FSH-S-11, $^{1}/_{15,600}$ dilution (stock)

GH: NIDDK r-GH-S-4, $^{1}/_{12,000}$ dilution (stock)

II.5 Timetable of radioimmunoassay

Day 1	All assays:	200 µl sample/standards (final dilutions in 0.01 M PBS/1% BSA)
		200 µl 0.01M PBS/1% BSA
		200 µl antiserum in buffer all dilutions in 0.1 M PBS/1% BSA
	LH and FSH:	Incubate at 4°C
	GH:	Incubate at room temperature.
Day 2	All assays:	200 µl ^{125}I-hormone in PBS/1% BSA
		~ 10,000 cpm for LH/FSH
		~ 12,000 cpm for FSH
		~ 6,000 cpm for GH
	LH and FSH:	Incubate at 4°C

	GH:	Incubate at room temperature after addition of label.
	GH only:	200 µl $^{1}/_{30}$ dilution of AHGG in 0.01 M PBS added approximately 7 h after label. Incubate at 4°C.
Day 3	GH only:	Centrifuge tubes (30 min at 2000 g, 4°C), aspirate supernatant and count pellet.
Day 4	LH and FSH:	200 µl of a $^{1}/_{20}$ dilution of ARGG in 0.01 M PBS. Incubate at 4°C.
Day 5	LH and FSH:	Centrifuge, aspirate and count.

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Lithium enhances the inhibitory effect of protein kinase C on LHRH-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*

By M. S. Johnson, D. J. MacEwan, R. Mitchell and F. J. Thomson *MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ*

The metabolism of inositol phosphates is modified by Li^+ (Allison *et al.* 1976), and it has been suggested that this may underlie the therapeutic effects of Li^+ in manic-depressive disorders (Berridge *et al.* 1982). However, agonist-induced diacylglycerol production is enhanced by Li^+ (Drummond & Raeburn, 1984), suggesting that Li^+ may modify several aspects of responses to Ca^{2+}-mobilizing receptors, including the activation of protein kinase C (PKC).

The influx of $^{45}Ca^{2+}$ into prisms of male rat anterior pituitary tissue was measured by an assay involving quenching of uptake after 30 s and extensive washing in ice-cold EGTA-containing medium (Fink *et al.* 1986). The $^{45}Ca^{2+}$ influx induced by 100 nM luteinizing hormone-releasing hormone (LHRH) but not by 300 nM thyrotrophin-releasing hormone (TRH) was inhibited by low concentrations (3–300 nM) of the PKC activator phorbol 12-myristate, 13-acetate (Mitchell *et al.* 1989). The concentration–response curve to LHRH but not TRH was biphasic, and the downturn at high LHRH concentrations was reversed by inhibitors of PKC (Fink *et al.* 1986). Therefore the mechanism of $^{45}Ca^{2+}$ influx induced by LHRH but not TRH can apparently be inhibited by hormone-induced activation of PKC. In the presence of LiCl (45 min pre-incubation), the $^{45}Ca^{2+}$ influx due to 100 nM-LHRH, but not 300 nM TRH or basal accumulation, was reduced, with 50% inhibition at $1{\cdot}0 \pm 0{\cdot}2$ mM (mean ± S.E.M., $n = 4$). This effect was reversed by the PKC inhibitor H7 (30 μM) but not by HA 1004 (Hidaka & Hagiwara, 1987). The biphasic concentration–response curve to LHRH was suppressed by 3 mM-Li^+ such that the maximal response was inhibited by approximately 50% and the downturn phase (from 100 to 1000 nM-LHRH) was amplified. It is possible that enhanced activation of PKC, as well as modified inositol phosphate metabolism, may contribute to the therapeutic actions of Li^+ *in vivo*.

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Effects of calmodulin antagonists on neurohormone-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*

By M. S. Johnson, D. J. MacEwan, R. Mitchell and F. J. Thomson. *MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ*

Luteinizing hormone-releasing hormone (LHRH) and thyrotrophin-releasing hormone (TRH) both induce $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue, but utilize different routes, which are regulated differentially by protein kinase C (Mitchell *et al.* 1989*b*). The $^{45}Ca^{2+}$ influx induced by LHRH is largely sensitive to nimodipine (suggesting that it may occur indirectly through voltage-sensitive Ca^{2+} channels), whereas the response to TRH is resistant to blockers of the known voltage-sensitive Ca^{2+} channels or of the Na^+/Ca^{2+} exchanger (Mitchell *et al.* 1989*b*). Receptor-activated Ca^{2+} entry (perhaps such as the response to TRH here) may involve a synergistic action of inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) and inositol 1,4,5-trisphosphate ($InsP_3$) (A. P. Morris *et al.* 1987). The production of $InsP_4$ from $InsP_3$ by a 3-kinase may be calmodulin (CM)-dependent (A. J. Morris *et al.* 1987). We have therefore investigated effects of CM antagonists on neurohormone-induced Ca^{2+} entry.

The rapid influx of $^{45}Ca^{2+}$ into prisms of tissue from male rats was measured in 30 s incubations with stimuli, as described previously (Mitchell *et al.* 1989*a*). At this time point, the stimulus-induced influx had reached a maximal increment over the basal $^{45}Ca^{2+}$ accumulation in each case. The influx of $^{45}Ca^{2+}$ induced by LHRH (100 nM) or TRH (100 nM), but not by 60 mM-K^+ medium, was inhibited by a new selective CM antagonist, 5-iodo-1-C_8 (MacNeil *et al.* 1988) at 1–30 μM. The related, but less selective compounds W7 and W5 (MacNeil *et al.* 1988) had similar effects with lower potencies. At concentrations of 100 μM, W7 and W5 also caused significant reduction of K^+-induced $^{45}Ca^{2+}$ influx (as did pimozide at just 10 μM), indicating that the specificity of these compounds is uncertain at such concentrations. The present results suggest that an unknown CM-dependent step is indeed involved in neurohormone-induced $^{45}Ca^{2+}$ influx regardless of whether this occurs via known voltage-sensitive Ca^{2+} channels (in the case of LHRH) or via some other route of receptor-activated Ca^{2+} entry (in the case of TRH).

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effect on either LH or FSH secretion). Phorbol 12,13-dibutyrate (PDBu) was kept in the dark as a stock solution in DMF at 1 mM. Stock solutions of 1-(5-isoquinolinesulphonyl)-2-methyl-piperazine hydrochloride (H7) (Gibco, Paisley, UK) were prepared in distilled water at a concentration of 10 mM and kept at −20°C. Luteinizing hormone-releasing hormone was from Peninsula Laboratories Europe (St. Helens, Merseyside, UK) and was used from an aliquoted stock solution (10 μg/ml) in 0.9% (w/v) NaCl kept at −40°C. Ionomycin (Novabiochem, Nottingham, UK), genistein (ICN Biochemicals, Cleveland, OH, USA) and 5-iodo-C8; *N*-(8-aminooctyl)-5-iodonaphthalene-1-sulphonamide (a gift from G.M. Blackburn and S. MacNeil, University of Sheffield, UK) were used from stock solution of 10 mM in DMF. Calmidazolium (R24571, stock 1 mM, DMF) and 48/80 (stock 10 mg/ml, H_2O) were obtained from Sigma Chemical Co. Finally, ML-7 (Novabiochem) was diluted from a stock of 10 mM in dilute acetic acid.

Statistics

Data are expressed as means ± SEM. When appropriate, statistical analyses were carried out using the Student's *t*-test or the Mann-Whitney *U*-test. The IC_{50} values for the kinase inhibitors were obtained from fitting the data with the non-linear, error-weighted iterative curve fitting program, P.fit (Biosoft, Cambridge, UK).

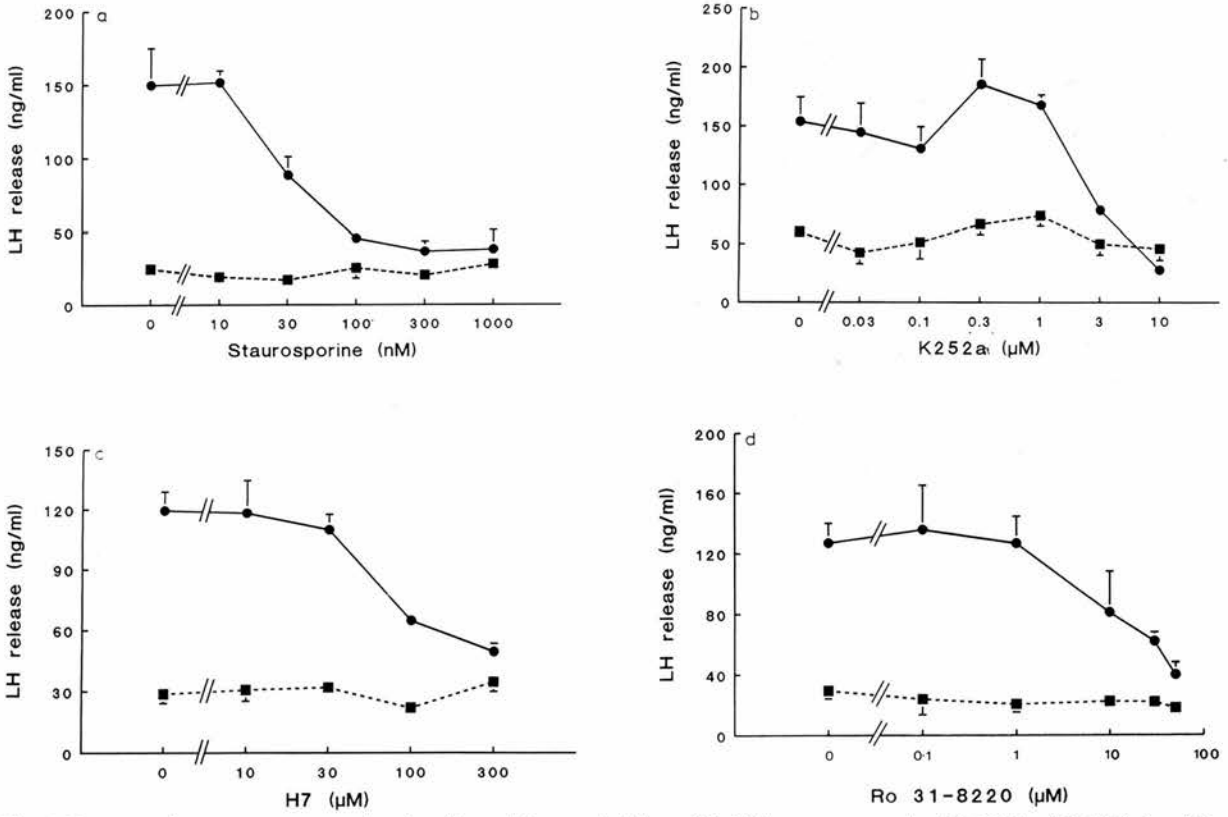

Fig. 1. Concentration–response curves for the effect of the protein kinase C inhibitors staurosporine (*a*), K252a (*b*), H7 (*c*) and Ro 31-8220 (*d*) on LHRH-induced release of LH. The squares (■) represent the release of gonadotropins from proestrous hemipituitaries in the 1st h of exposure to LHRH (0.85 nM) and the circles (●) release in a 2nd h of exposure to the peptide. The greater release in the 2nd h is a result of LHRH priming, which, unlike the initial release effected by this peptide, is inhibited by staurosporine. Values are mean ± SEM and each point is $4 \leq n \geq 6$.Corresponding basal release of LH was between 5.2–14.8 ng/ml and was unaffected by any of the inhibitors or their vehicles at the concentrations used.

Results

Consecutive hourly incubations of proestrous hemipituitaries with LHRH (0.85 nM) induced a relatively small release of both LH and FSH in the 1st h, followed by augmented release of gonadotropin during a 2nd h with LHRH, due to the self-priming effect of LHRH (Figs. 1 and 2). Inclusion of staurosporine (10 nM–2 μM), which by itself had no effect on basal gonadotropin release over several hours, had no effect on the release of gonadotropins seen in the 1st h of incubation with LHRH (see Figs. 1*a* and 2*a*). However, exposure to staurosporine caused a concentration-dependent inhibition of the greater release of gonadotropins brought about by priming. Luteinizing hormone release in the 2nd h with LHRH was significantly decreased ($p < 0.05$) compared to control at concentrations of 30 nM staurosporine and above; results were similar for FSH. The concentration of staurosporine at which 50% inhibition (IC_{50}) occurred was 26.3 ± 7.0 nM for LH and 37.0 ± 7.1 nM for FSH. In the presence of staurosporine at concentrations of 300 nM and above, release of both LH and FSH was decreased to levels seen in the 1st h with LHRH but not to the basal levels of release. Table 1 shows the effect of staurosporine on LHRH-induced gonadotropin release from anterior pituitary tissue obtained on different days of the estrous cycle. The magnitude of the priming effect of LHRH was dependent on the stage of the estrous cycle, being largest on proestrus and smallest on estrus (as described by Aiyer et al.,

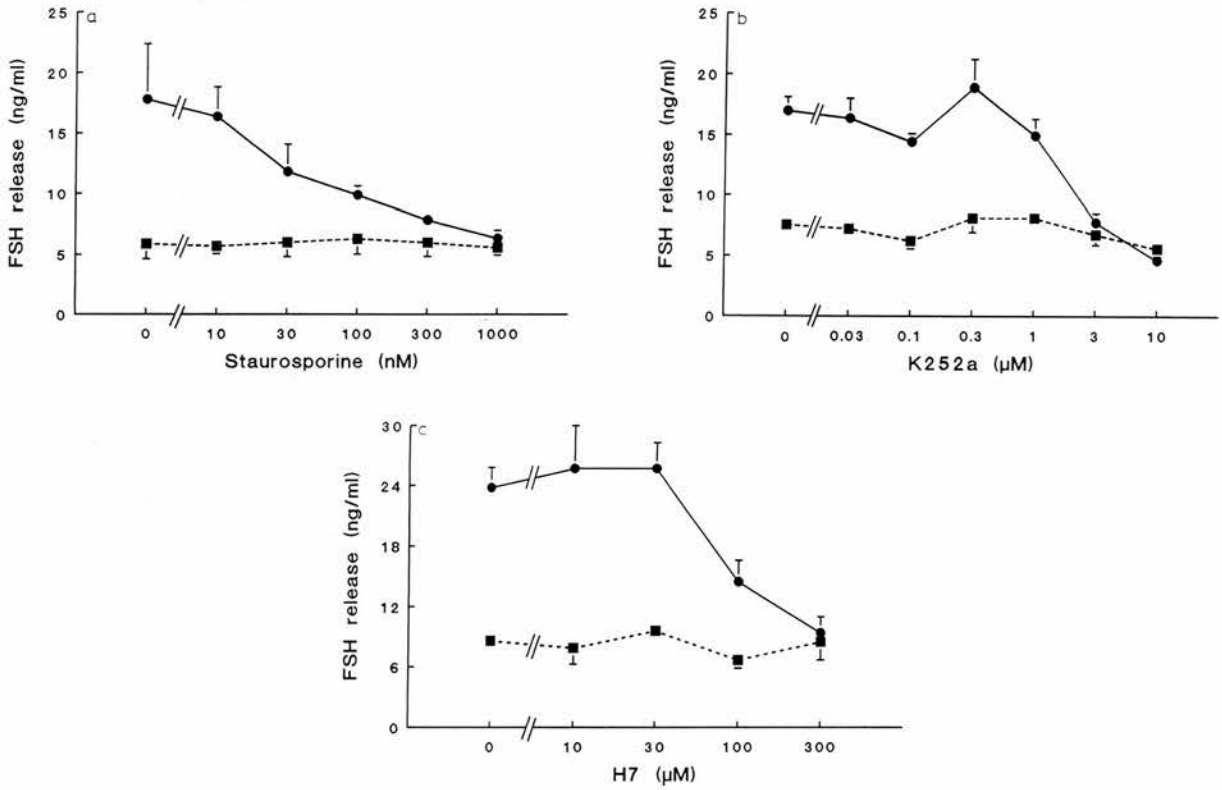

Fig. 2. Concentration–response curves for the effect of staurosporine (*a*), K252a (*b*) and H7 (*c*) on LHRH-induced release of FSH. The squares (■) represent the release of gonadotropins from proestrous hemipituitaries in the 1st h of exposure to LHRH (0.85 nM) and the circles (●) release in a 2nd h of exposure to the peptide. The greater release in the 2nd h is the result of the LHRH-priming effect which, unlike the release in the 1st h with LHRH, is inhibited by K252a. Values are mean ± SEM and at each point $4 \leq n \geq 6$. Corresponding basal release of FSH was between 3.3–4.6 ng/ml and was unaffected by any of the inhibitors or their vehicles at the concentrations used.

TABLE 1

EFFECT OF STAUROSPORINE ON LHRH-INDUCED LH RELEASE OVER THE DAYS OF THE ESTROUS CYCLE AND IN MALES

Hemipituitaries were incubated in vitro for 3 consecutive hours. In the basal hour either medium alone (controls) or staurosporine (300 nM) was present. In the following 2 h there was, in addition, LHRH (0.85 nM). Values given are the mean ± SEM.

Days of cycle	Inhibitor	LH (ng/ml)			*n*
		Basal hour	1st hour	2nd hour	
Estrus	Control	8.4 ± 1.6	14.2 ± 1.7	35.9 ± 2.3	4
	Staurosporine	6.2 ± 0.7	9.5 ± 1.1	9.2 ± 1.1 **	4
Metestrus	Control	4.9 ± 1.2	5.3 ± 0.4	25.1 ± 2.2	4
	Staurosporine	6.1 ± 1.4	4.6 ± 1.0	5.8 ± 1.3 **	4
Diestrus	Control	5.25 ± 1.17	8.63 ± 0.99	55.70 ± 4.94	6
	Staurosporine	2.92 ± 0.56	4.63 ± 0.37	6.15 ± 0.56 **	6
Proestrus	Control	5.25 ± 0.67	27.03 ± 2.59	159.7 ± 14.43	6
	Staurosporine	6.58 ± 0.78	25.4 ± 3.03	30.70 ± 4.26 **	6
Males	Control	6.10 ± 1.40	10.15 ± 1.77	21.03 ± 4.85	4
	Staurosporine	8.04 ± 1.04	8.70 ± 0.69	5.74 ± 0.45 *	4

* $p < 0.05$ significantly different compared to control.
** $p < 0.01$ significantly different compared to control.

TABLE 2

EFFECTS OF SOME PROTEIN KINASE INHIBITORS ON LHRH-INDUCED LH AND FSH RELEASE AND PRIMING

Hemipituitaries were incubated in vitro for 3 h. In the basal hour there was medium alone (control) or a protein kinase inhibitor. In hours 1 and 2 there was, in addition, LHRH (0.85 nM). Values given are mean ± SEM.

Inhibitor	LH (ng/ml)			*n*
	Basal hour	1st hour	2nd hour	
Control	20.7 ± 2.8	81.9 ± 8.3	285.1 ± 21.2	8
Genistein (50 μM)	14.0 ± 3.3	79.0 ± 14.8	336.3 ± 35.2	4
Control	8.5 ± 1.8	36.2 ± 8.2	191.7 ± 26.7	6
5-Iodo-C8 (10 μM)	15.6 ± 3.0	92.0 ± 11.7 **	282.5 ± 20.2 *	6
Control	7.7 ± 1.7	123.4 ± 13.5	283.0 ± 6.0	5
48/80 (30 μg/ml)	10.6 ± 2.6	184.7 ± 10.9 *	299.5 ± 11.2	5
Control	4.2 ± 0.6	37.7 ± 5.0	134.3 ± 12.0	5
Calmidazolium (1 μM)	5.8 ± 1.5	71.0 ± 11.8 *	215.6 ± 22.4 *	6
Control	7.9 ± 3.1	46.9 ± 6.3	159.0 ± 12.7	4
ML-7 (3 μM)	4.2 ± 0.5	57.0 ± 5.4	212.7 ± 26.8	4
	FSH (ng/ml)			
	Basal hour	1st hour	2nd hour	
Control	5.01 ± 0.29	9.93 ± 0.96	25.47 ± 3.52	8
Genistein	4.29 ± 0.44	7.75 ± 0.97	22.59 ± 3.97	4
Control	3.55 ± 0.38	5.89 ± 0.64	17.76 ± 2.29	4
5-Iodo-C8	5.08 ± 0.34	10.27 ± 0.82 *	28.57 ± 3.28	6
Control	2.63 ± 0.28	10.11 ± 2.38	41.72 ± 1.23	5
48/80	4.18 ± 0.47	18.16 ± 0.94 *	45.83 ± 3.62	5
Control	2.76 ± 0.23	7.50 ± 1.15	17.85 ± 2.09	4
ML-7	2.68 ± 0.19	8.49 ± 0.59	25.09 ± 2.29 *	4

* $p < 0.05$ significantly different compared to control.
** $p < 0.01$ significantly different compared to control.

1974b and Table 1). At each stage of the cycle, staurosporine (300 nM) blocked LHRH-induced gonadotropin release in the 2nd h but only to a level equivalent to that seen in the 1st h of LHRH incubation.

The less active congener of staurosporine K252a (30 nM–10 μM) also caused a concentration-dependent inhibition of the 2nd h of LHRH-induced gonadotropin release (Figs. 1*b* and 2*b*) again without affecting initial gonadotropin release. There was a significant decrease ($p < 0.05$) in LHRH-induced release of both LH and FSH compared to control at K252a concentrations of 3 μM and above. The IC_{50} for inhibition of LHRH-induced release by K252a was 2.9 ± 0.4 μM for LH and was 2.3 ± 0.5 μM for FSH.

Figs. 1*c* and 2*c* show the effect on LHRH priming of a PKC inhibitor of quite different structure, H7 (10–300 μM). At concentrations above 30 μM H7 there was significant ($p < 0.05$) inhibition of both LH and FSH release in the 2nd h with LHRH, with no effect on LHRH-induced gonadotropin release in the 1st h. The IC_{50} for inhibition of the 2nd h of LHRH-induced release by H7 was 71.6 ± 13.3 μM for LH and 88.1 ± 20.4 μM for FSH. The novel and highly-selective PKC inhibitor, Ro 31-8220 (0.1–50 μM), also caused a concentration-dependent inhibition of the primed but not the unprimed gonadotropin secretion induced by LHRH (Fig. 1*d*). At concentrations of 10 μM and above, the inhibition was statistically significant ($p < 0.05$) and gave an IC_{50} for inhibition of the 2nd h of LHRH-induced LH release of 19.0 ± 6.8 μM.

A range of inhibitors of different classes of kinases (used at concentrations described in the literature to cause more than 50% inhibition of the relevant kinase in whole cells) were compared (Table 2). Genistein (50 μM), an inhibitor of several tyrosine kinases (Akiyama et al., 1987), had no effect on either initial LHRH-induced gonadotropin release or priming. Calmidazolium (R24571), 48/80 (Gietzen, 1983) and 5-iodo-C8 (MacNeil et al., 1988), which inhibit Ca^{2+}/calmodulin-dependent kinases, all significantly increased LHRH-induced release of LH and FSH in either the 1st, 2nd or both hours with LHRH, but did not block the priming effect. Similarly, ML-7 which is a selective inhibitor of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) acting at a site other than the calmodulin recognition site (Saitoh et al., 1987), failed to block LHRH priming.

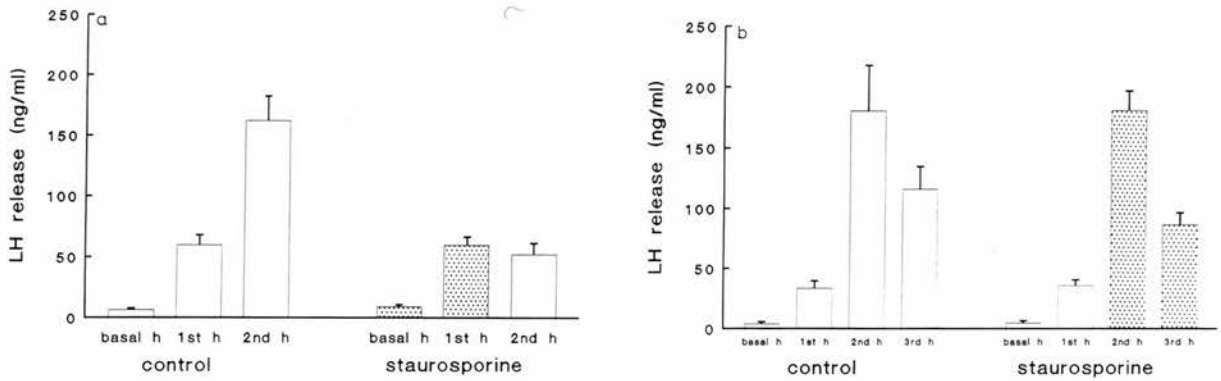

Fig. 3. Effect of staurosporine on the induction/expression of LHRH priming. Release of LH from proestrous hemipituitaries was measured over either 2 or 3 consecutive hours with LHRH (0.85 nM). In part *a*, staurosporine (300 nM) was included (dotted bars) in the basal h and 1st h of LHRH (i.e. that hour during which priming takes place). Between the 1st and 2nd h with LHRH, the tissue was washed twice with MEM to remove the staurosporine before incubating the tissue with LHRH alone for a 2nd h. Although staurosporine was not present in the 2nd h, primed release was significantly inhibited ($p < 0.05$). Part *b* shows the effect of staurosporine (300 nM) on LHRH-stimulated LH release from previously-primed proestrous tissue. Hemipituitaries were primed by incubating with LHRH for 1 h. They then received a further 2 consecutive hours of incubation in the presence of LHRH and 300 nM staurosporine (dotted bars). Staurosporine was unable to inhibit LHRH-stimulated LH release from previously-primed tissue. Values are mean $\pm$ SEM ($n = 4$).

In order to determine whether the staurosporine-sensitive step was in the induction of the priming effect rather than its expression, several experiments were carried out. Firstly, staurosporine (300 nM) was included in the basal and 1st h of LHRH incubation (i.e. that hour during which priming takes place), after which staurosporine was removed and the tissue was extensively washed before a second hourly incubation in the presence of LHRH only (see Fig. 3*a*). As observed previously, staurosporine did not alter initial LHRH-induced LH release. However, LHRH-induced release of LH in the 2nd h of LHRH was still fully blocked to a level equivalent to that in the 1st h responses to LHRH. Two pieces of evidence indicated that this washing procedure was successful in substantially reducing the staurosporine concentration in the tissue: (i) an identical procedure using [^{3}H]*N*,*N*-dimethyl staurosporine (a close structural analogue of staurosporine) resulted in a reduction of the tissue concentration to $8 \pm 2\%$ ($n = 4$) of the initial level; (ii) in tissue incubated with staurosporine and washed as above, the facilitation of K^+-induced $^{45}Ca^{2+}$ influx caused by 300 nM PDBu (MacEwan and Mitchell, 1991) was not reduced from that in control tissue ($108 \pm 10\%$ of the facilitation in vehicle-incubated controls, $n =$ 3). In the second series of experiments we investigated whether staurosporine affected LHRH-induced LH release from previously-primed tissue (Fig. 3*b*). In this experiment, tissue was primed with LHRH for 1 h before including staurosporine (300 nM) during the 2nd and 3rd h LHRH incubations. Even over a period of 2 h (to allow for the slow development of any possible effect) staurosporine did not block LHRH-induced gonadotropin release from previously-primed tissue. The results of these two experiments show clearly that staurosporine blocks the priming but not the gonadotropin-releasing action of LHRH. The time course of the action of staurosporine is shown in Table 3. When staurosporine was added over four 30 min incubation periods with LHRH, the increment in LH release due to priming seen in periods 2, 3 and 4 of control (LHRH alone) incubations was abolished completely. However, when staurosporine was added later, at the beginning of periods 2 or 3, primed release was progressively less inhibited. The ability of LHRH priming to escape inhibition by staurosporine if the tissue is exposed to LHRH for 30 min prior to the inhibitor is in agreement with data using the protein synthesis inhibitor cycloheximide, which also prevents LHRH priming with similar temporal characteristics (Picker-

TABLE 3

EFFECT OF STAUROSPORINE ON THE TIME COURSE OF LHRH-INDUCED LH RELEASE

Release of LH was measured from hemipituitaries that were incubated in vitro for a basal 30 min and then four consecutive 30 min periods with LHRH (0.85 nM). The basal release of LH was stripped from the values for LHRH-induced LH release in the time periods 1–4. The PKC inhibitor, staurosporine (300 nM) was added into the incubation series at the beginning of the time period at which + is indicated in the row above the data and was present to the end of the experiment. Values given are mean ± SEM ($n = 4$).

	LH (ng/ml)			
	30 min incubations			
	1	2	3	4
Staurosporine	–	–	–	–
LHRH	17.6 ± 10.5	52.7 ± 14.5	104.6 ± 10.2	137.2 ± 16.4
Staurosporine	–	–	+	+
LHRH	6.8 ± 3.4	35.8 ± 2.9	84.9 ± 8.6	76.5 ± 3.9 *
Staurosporine	–	+	+	+
LHRH	8.2 ± 3.7	30.7 ± 11.4	57.2 ± 17.1	62.9 ± 20.4 **
Staurosporine	+	+	+	+
LHRH	17.3 ± 7.0	25.5 ± 9.8	20.8 ± 8.3 **	20.5 ± 8.4 **

* $p < 0.05$ significantly different compared to control (LHRH alone).
** $p < 0.01$ significantly different compared to control (LHRH alone).

ing and Fink, 1976). Some priming still occurred if pituitaries were exposed to LHRH for as little as 15 min before a concentration of cycloheximide was added that was sufficient to abolish priming if given in conjunction with LHRH.

To confirm that staurosporine has no inhibitory activity upon the secretory apparatus of the cell, we investigated the effect of this drug on ionomycin (50 μM)-induced release of LH and FSH. In the presence of 300 nM staurosporine, there was clearly no significant alteration in LH or FSH release induced by a 1st h or 2nd h incubation with ionomycin (Table 4). In the final experiment hemipituitaries were primed with LHRH (0.85 nM) either in the presence or absence of staurosporine (300 nM) and ionomycin (30 μM) was used as a receptor-independent secretagogue to elicit primed LH release. After priming with LHRH, ionomycin induced a much greater release of LH (157 ± 11 ng/ml) than in control incubations with ionomycin alone (63 ± 6 ng/ml). The addition of staurosporine during the priming hour, however, fully prevented this facilitation of the response to ionomycin (LH release of 69 ± 4 ng/ml; $n = 4$–6).

Discussion

The results presented here indicate that activation of a protein kinase, probably a form of PKC, is necessary for LHRH priming but not initial LHRH-induced gonadotropin release. Thus, the facilitation of gonadotropin release brought about by LHRH priming was completely prevented by staurosporine, leaving LHRH-induced release equivalent to that of unprimed responses, regardless of the degree of priming that had occurred (Table 1). The absolute potency of staurosporine here (26.3 ± 7.0 nM) is similar to that reported in thoroughly-characterised PKC-mediated cellular responses, for example, in platelets, T cells and spinal afferent nerve terminals (Davis et al., 1989; Dunn and Rang, 1990) and in cell-free assays of the activity of partially- or extensively-purified PKCs (Nakadate et al., 1988; Schaap and Parker, 1990; Johnson and Mitchell, unpublished). Indeed, under equivalent conditions to the present experiments, the release of LH elicited by 100 nM PDBu was similarly inhibited by staurosporine with a IC_{50} of 44.0 ± 17.2 nM ($n = 4$). The potency ratio for the inhibitory effects of staurosporine and K252a on LHRH priming (62- and 111-fold for FSH and LH respectively) is of a similar order to that reported for their inhibition of PKC in cell-free systems (36-fold) but contrasts with the corresponding values for cAMP-dependent (PKA) and cGMP-dependent (PKG) protein kinases of 2.5 and 2.3 respectively (Kase et al., 1986; Tamaoki et al., 1986). A novel PKC inhibitor, Ro 31-8220 (which is reported to show selectivity for PKC over PKA and Ca^{2+}/calmodulin-dependent kinase of 2–3 orders of magnitude; Davis et al., 1989), also inhibited LHRH priming of LH release, with an IC_{50} value of 19.0 ± 6.8 μM, similar to that for inhibition of PDBu (300 nM)-induced LH release (15.6 ± 4.5 μM, $n = 5$). The isoquinoline inhibitor of PKC, H7, also inhibited LHRH priming. There was, however, an interesting disparity between the IC_{50} of H7 on LHRH priming (71.6 ± 13.3 μM) and on PDBu (100 nM)-induced LH release (1.7 ± 1.5 μM, $n = 6$). We have previously described several other instances where phorbol ester-induced effects were inhibited with unusually low potency of H7 but not of staurosporine (Johnson and Mitchell, 1989; Johnson et al., 1989; Fink et al., 1990; MacEwan and Mitchell, 1991; Thomson et al., 1991a) and the ability of staurosporine but not H7 to inhibit some phorbol ester effects has also been described by others (Watson et al., 1988; Nakadate et al., 1989). Indeed when the PDBu-induced kinase activity of PKCs partially purified from anterior pituitary but not brain tissue was measured in a mixed micelle assay (Johnson et al., 1991), a Ca^{2+}-independent component of the activity was apparent which displayed a reduced potency towards H7 but not staurosporine. Both Ca^{2+}-dependent and Ca^{2+}-independent PDBu-induced activity were inhibited by staurosporine with similar IC_{50} values (114 ± 65 nM and 107 ± 41 nM respectively), but H7 inhibited Ca^{2+}-dependent activity (9 ± 4 μM) much more effectively than Ca^{2+}-independent activity (47 ± 20 μM). In brain and a number of peripheral tissues, staurosporine and H7 both displayed identical IC_{50} values on Ca^{2+}-dependent and -independent activity.

Since both staurosporine and H7 are considered to act close to the ATP-binding site of PKCs, which is quite highly conserved in a number of kinases, such disparities in the potency of H7 but not staurosporine are at first sight unexpected. However, the sites of action of these inhibitors appear to be non-identical. While K252a has been reported to be a competitive inhibitor with respect to ATP (Kase et al., 1987) there is evidence to the contrary for staurosporine (Tamaoki et al., 1986; Rüegg and Burgess, 1989) and the binding of [^{3}H]*N*,*N*-dimethyl staurosporine is displaced with only extremely low potency by H7 ($IC_{50} > 500$ μM) (Thomson et al., 1991b). Although kinase inhibitors of the isoquinoline type do appear to act in a kinetically competitive manner with respect to ATP (Hidaka et al., 1984), certain analogues (for example HA 1004 and H7) demonstrate distinct relative selectivity for or against particular kinases (Hidaka and Hagiwara, 1987) indicating that a degree of recognition specificity is possible at this site. Furthermore, it is not clear that the H7 recognition site is entirely concurrent with that for ATP, since H7 provides only partial protection against denaturation of the ATP site by covalent chemical reagents (Ohta et al., 1988). Finally, in some cases, the relationship between H7 and ATP recognition may be complex since the sequences of α and β isoforms of PKC contain a second consensus recognition motif for ATP (Huang, 1989). It is unclear whether the relatively H7-resistant kinase involved in LHRH priming might represent a particular PKC isozyme, some modified form of PKC, a distinct but related kinase or the influence of other as yet quite unknown factors. Whichever is the case, the consensus of inhibition of LHRH priming by four PKC inhibitors, taken with the ineffectiveness of an array of inhibitors of other types of kinase, strongly suggests that a form of PKC or related kinase is required for LHRH priming.

Staurosporine and K252a can have inhibitory actions on tyrosine kinases (Yamaguchi and Kathuria, 1988), PKA (Tamaoki et al., 1986), myosin light chain kinase and Ca^{2+}/calmodulin-dependent (CaM II) kinase (Watson et al., 1988). However, the action of staurosporine on LHRH priming seems unlikely to involve these kinases since inhibitors of tyrosine kinase (genistein), CaM II kinase (the calmodulin antagonists: calmidazolium, 5-iodo-C8 and 48/80) and MLCK (ML-7) were ineffective and both the potency ratio of staurosporine versus K252a on LHRH priming and the identical potency of Ro 31-8220 on priming and PDBu-induced responses argue against a role of cyclic nucleotide-dependent kinases. Interestingly, both initial LHRH-induced release and/or that from primed tissue were enhanced by a number of the CaM II kinase inhibitors. Evidence supporting a role of CaM II kinase in LHRH-induced gonadotropin release has been provided by several laboratories (Conn et al., 1984; Das et al., 1989) but recent evidence (Van der Merwe et al., 1990b) suggests that it is only the more non-specific of the calmodulin antagonists used that inhibit LHRH-induced LH release. Certainly raised intracellular Ca^{2+} is an effective secretagogue for LH, yet it will not mimic the priming effect of LHRH (Table 4). Interestingly, removal of extracellular Ca^{2+} during an initial hour of incubation with LHRH, while reducing LH release in that hour, does not prevent LHRH priming if Ca^{2+} is replaced in a second hour with LHRH (Pickering and Fink, 1979). If CaM II kinase is involved in the first phase of LHRH-induced release it is unclear why our results show an increase of LH release in the

TABLE 4

EFFECT OF STAUROSPORINE ON IONOMYCIN-INDUCED LH AND FSH RELEASE

Hemipituitaries were incubated in vitro for 3 h. In the basal hour there was medium alone (controls) or staurosporine (300 nM). In the next 2 consecutive hours there was, in addition, ionomycin (50 μM). Values given are the mean ± SEM ($n = 7$).

Inhibitor	LH (ng/ml)		
	Basal hour	1st hour	2nd hour
Control	5.5 ± 1.0	95.5 ± 13.8	63.1 ± 12.8
Staurosporine (300 nM)	6.1 ± 0.4	108.6 ± 13.0	61.9 ± 8.7
	FSH (ng/ml)		
	Basal hour	1st hour	2nd hour
Control	2.47 ± 0.16	8.65 ± 0.90	6.52 ± 0.67
Staurosporine (300 nM)	2.45 ± 0.14	7.66 ± 0.63	5.27 ± 0.38

presence of CaM II kinase inhibitors. The Ca^{2+}/calmodulin-dependent phosphatase, calcineurin (Armstrong, 1989) has been detected in pituitary cells (Farber et al., 1987), so if raised Ca^{2+} levels in gonadotropes were to increase both calmodulin-dependent phosphorylations and dephosphorylations then interpretation of the overall effects of calmodulin antagonists would be complex.

Staurosporine exerts its inhibitory effects on primed gonadotropin release if present during that hour in which LHRH priming takes place (i.e. the 1st h), but is removed for the hour of further challenge with LHRH, showing that its action is on the induction rather than the expression of LHRH priming (Fig. 3*a*). Furthermore, the response of previously-primed tissue to LHRH was unaltered by staurosporine (Fig. 3*b*). Since staurosporine will also prevent the facilitated response to ionomycin seen in previously-primed tissue, staurosporine cannot simply be acting as a slowly-acting inhibitor of a late component of LHRH responses. Additional evidence that staurosporine was not acting by a delayed, non-specific action on the secretory apparatus of the gonadotrope was provided by its lack of effect alone on secretion induced by the Ca^{2+} ionophore, ionomycin (Table 3). In an analogous fashion to its role here in LHRH priming, PKC has been implicated in the induction but not expression of another phenomenon of increased cellular responsiveness, long-term potentiation (LTP) of synaptic transmission in hippocampus (Malinow et al., 1989). The induction of LTP can be prevented by selective peptide inhibitors of CaM II kinase as well as by PKC inhibitors (Malinow et al., 1989), but it is not yet possible to say with any certainty whether such factors, in addition to PKC, serve any role in LHRH priming.

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The priming effect of luteinizing hormone-releasing hormone (LHRH) but not LHRH-induced gonadotropin release, can be prevented by certain protein kinase C inhibitors

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Key words: Gonadotropin; Luteinizing hormone-releasing hormone priming; Protein kinase C; Staurosporine

Summary

The priming effect of LHRH in vitro (which results in increased responsiveness of gonadotropes to both LHRH receptor-mediated and receptor-independent stimuli) is brought about by an unknown mechanism. The present results indicate that induction of the LHRH priming effect is inhibited in a concentration-dependent manner by the protein kinase C (PKC) inhibitors staurosporine, K252a, H7 and by the novel highly-selective PKC inhibitor, Ro 31-8220. In contrast, a range of other compounds that are relatively selective inhibitors of other kinases such as tyrosine kinases and Ca^{2+}/calmodulin-dependent kinases were unable to prevent priming. The PKC inhibitors prevented priming without affecting initial LHRH-induced gonadotropin secretion. Thus, the priming-elicited increment in secretion was selectively removed, restoring hormone release to the level measured during an initial response to LHRH. Similar results were obtained on different days of the estrous cycle where the magnitude of the priming effect varies. Experiments on the time course of PKC inhibitor action revealed that the critical period was in the induction of the priming effect, not its expression. The PKC inhibitors had neither acute nor delayed effects on gonadotropin secretion induced by ionomycin. Staurosporine, K252a and Ro 31-8220 inhibited LHRH priming with identical potencies to their inhibition of phorbol ester-induced gonadotropin secretion. The reduced potency of H7 seen on LHRH priming compared to phorbol ester-induced gonadotropin release parallels results seen with this inhibitor on phorbol ester-induced secretion of growth hormone (Johnson and Mitchell (1989) Biochem. Soc. Trans. 17, 751–752) and on the pharmacological characteristics of PKCs partially purified from anterior pituitary tissue. In all aspects of this study, effects on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion appeared to be entirely similar.

Introduction

Luteinizing hormone-releasing hormone (LHRH) has the ability to increase the responsiveness of gonadotropes to itself; an action known as the priming effect of LHRH (Aiyer et al., 1974). As a result, the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in response to LHRH is greatly enhanced after an initial exposure to the peptide hormone. The mechanism by which this increase in pituitary responsiveness to LHRH occurs is unknown

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but the effect is dependent both on protein synthesis and the integrity of microfilaments (Pickering and Fink, 1979). We have previously shown that a facilitation of LHRH-induced second messenger production (and subsequent intracellular events) occurs in LHRH priming (Mitchell et al., 1988). In addition, the priming effect involves a general facilitation of stimulus-secretion coupling in gonadotropes such that receptor-independent secretagogues will also elicit greater release of gonadotropins after LHRH priming (Pickering and Fink, 1979; Johnson and Mitchell, 1991). The present experiments were carried out in order to clarify the second messenger pathways involved in the induction of the priming effect.

Activation of LHRH receptors on gonadotropes results in phosphoinositide hydrolysis (Schrey, 1985; Andrews and Conn, 1986), and so it seems likely that the priming phenomenon is dependent on production of either inositol 1,4,5-trisphosphate (IP_3) or diacylglycerol (DAG) (which lead to release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC) respectively). There is considerable evidence for Ca^{2+} having a major role in LHRH-induced gonadotropin release (see Huckle and Conn, 1988, for review). In contrast, the consensus (McArdle et al., 1987; Johnson et al., 1988; Van der Merwe et al., 1990a) but not all (Stojilkovic et al., 1988) reports indicate little involvement of PKC in acute LHRH-induced gonadotropin release. We previously described that although PKC activation could augment the response to LHRH in a slowly-developing manner analogous to priming, it did not fully pre-empt priming elicited by LHRH. Furthermore, the PKC inhibitor H7 (Hidaka and Hagiwara, 1987) had no apparent effect on LHRH priming at a concentration (10 μM) which effectively reversed the effects of phorbol esters on gonadotropin release (Johnson et al., 1988). However, we now report that the PKC inhibitor staurosporine (Tamaoki et al., 1986), its congener K252a (Kase et al., 1986), the novel highly-selective PKC inhibitor Ro 31-8220 (Davis et al., 1989), and somewhat higher concentrations of H7 are able to inhibit LHRH priming without affecting initial LHRH-induced gonadotropin release.

Materials and methods

The methods and experimental design were based on those described by Pickering and Fink (1976) with some modifications. Female COB Wistar rats (200–250 g; Charles River UK) were maintained under controlled conditions with free access to food pellets (CRM, Labsure, Manea, Cambs., UK) and water. Vaginal smears were examined for at least two regular 4-day estrous cycles before sacrificing the rats at 13.30 h on the appropriate day of the estrous cycle. Pituitary glands were removed and the anterior lobes separated and hemisected. These hemipituitaries were allocated one/flask such that hemipituitaries from the same rat were in different treatment groups. They were incubated in 2 ml of prewarmed and gassed (95% O_2/5% CO_2) Hepes-buffered minimal essential medium (MEM) with Earle's salts at 37°C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2. After a preincubation of 30 min, and every subsequent hour thereafter, the medium was changed for fresh MEM. Replacement medium contained either a kinase inhibitor or no drug for the initial basal hour and then, in addition, LHRH (0.85 nM) or ionomycin (50 μM) in the subsequent hours (1st, 2nd, and 3rd h). To investigate the effect of staurosporine on the time course of the development of priming, the incubation periods were reduced to 30 min, and there were five consecutive incubations.

The medium removed at the end of each incubation period was kept at −40°C until assayed for LH and FSH by radioimmunoassay (Niswender et al., 1968; Daane and Parlow, 1971) as used previously in this laboratory (Johnson et al., 1988). The standards used were NIADDK-rat FSH-RP2 and NIH-LH-S18 for the majority of figures and tables, although NIADDK-rat LH-RP2 was used for the data in Table 1 and Fig. 3.

Drugs

Staurosporine, K252a (from Kyowa Medex Co., Tokyo, Japan) and Ro 31-8220 (Roche Products, Welwyn, UK) were made up as stock solutions at 2–10 mM in dimethylformamide (DMF) (the maximum concentration of DMF (0.5%, v/v) used was included in control experiments and had no

effect on either LH or FSH secretion). Phorbol 12,13-dibutyrate (PDBu) was kept in the dark as a stock solution in DMF at 1 mM. Stock solutions of 1-(5-isoquinolinesulphonyl)-2-methyl-piperazine hydrochloride (H7) (Gibco, Paisley, UK) were prepared in distilled water at a concentration of 10 mM and kept at −20°C. Luteinizing hormone-releasing hormone was from Peninsula Laboratories Europe (St. Helens, Merseyside, UK) and was used from an aliquoted stock solution (10 μg/ml) in 0.9% (w/v) NaCl kept at −40°C. Ionomycin (Novabiochem, Nottingham, UK), genistein (ICN Biochemicals, Cleveland, OH, USA) and 5-iodo-C8; *N*-(8-aminooctyl)-5-iodonaphthalene-1-sulphonamide (a gift from G.M. Blackburn and S. MacNeil, University of Sheffield, UK) were used from stock solution of 10 mM in DMF. Calmidazolium (R24571, stock 1 mM, DMF) and 48/80 (stock 10 mg/ml, H_2O) were obtained from Sigma Chemical Co. Finally, ML-7 (Novabiochem) was diluted from a stock of 10 mM in dilute acetic acid.

Statistics

Data are expressed as means ± SEM. When appropriate, statistical analyses were carried out using the Student's *t*-test or the Mann-Whitney *U*-test. The IC_{50} values for the kinase inhibitors were obtained from fitting the data with the non-linear, error-weighted iterative curve fitting program, P.fit (Biosoft, Cambridge, UK).

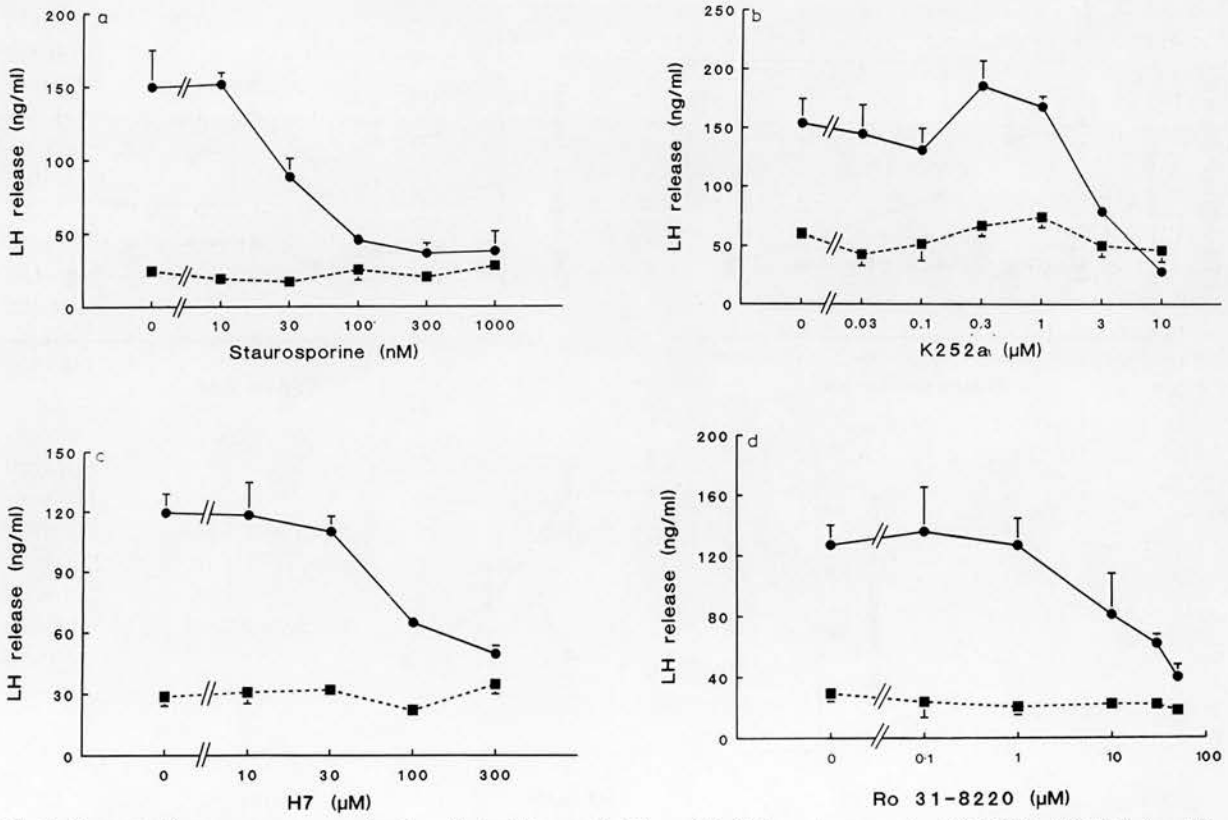

Fig. 1. Concentration–response curves for the effect of the protein kinase C inhibitors staurosporine (*a*), K252a (*b*), H7 (*c*) and Ro 31-8220 (*d*) on LHRH-induced release of LH. The squares (■) represent the release of gonadotropins from proestrous hemipituitaries in the 1st h of exposure to LHRH (0.85 nM) and the circles (●) release in a 2nd h of exposure to the peptide. The greater release in the 2nd h is a result of LHRH priming, which, unlike the initial release effected by this peptide, is inhibited by staurosporine. Values are mean ± SEM and each point is $4 \leq n \geq 6$.Corresponding basal release of LH was between 5.2–14.8 ng/ml and was unaffected by any of the inhibitors or their vehicles at the concentrations used.

Results

Consecutive hourly incubations of proestrous hemipituitaries with LHRH (0.85 nM) induced a relatively small release of both LH and FSH in the 1st h, followed by augmented release of gonadotropin during a 2nd h with LHRH, due to the self-priming effect of LHRH (Figs. 1 and 2). Inclusion of staurosporine (10 nM–2 μM), which by itself had no effect on basal gonadotropin release over several hours, had no effect on the release of gonadotropins seen in the 1st h of incubation with LHRH (see Figs. 1*a* and 2*a*). However, exposure to staurosporine caused a concentration-dependent inhibition of the greater release of gonadotropins brought about by priming. Luteinizing hormone release in the 2nd h with LHRH was significantly decreased ($p < 0.05$) compared to control at concentrations of 30 nM staurosporine and above; results were similar for FSH. The concentration of staurosporine at which 50% inhibition (IC_{50}) occurred was 26.3 $\pm$ 7.0 nM for LH and 37.0 $\pm$ 7.1 nM for FSH. In the presence of staurosporine at concentrations of 300 nM and above, release of both LH and FSH was decreased to levels seen in the 1st h with LHRH but not to the basal levels of release. Table 1 shows the effect of staurosporine on LHRH-induced gonadotropin release from anterior pituitary tissue obtained on different days of the estrous cycle. The magnitude of the priming effect of LHRH was dependent on the stage of the estrous cycle, being largest on proestrus and smallest on estrus (as described by Aiyer et al.,

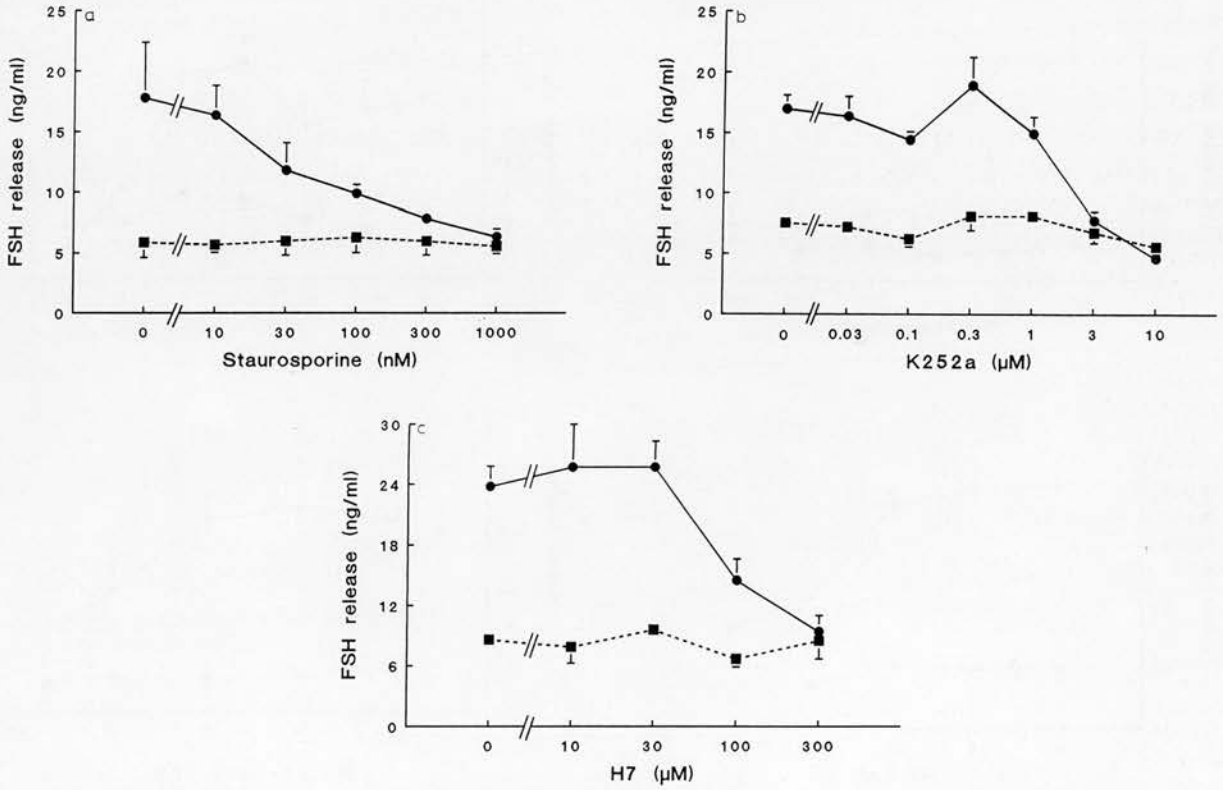

Fig. 2. Concentration–response curves for the effect of staurosporine (*a*), K252a (*b*) and H7 (*c*) on LHRH-induced release of FSH. The squares (■) represent the release of gonadotropins from proestrous hemipituitaries in the 1st h of exposure to LHRH (0.85 nM) and the circles (●) release in a 2nd h of exposure to the peptide. The greater release in the 2nd h is the result of the LHRH-priming effect which, unlike the release in the 1st h with LHRH, is inhibited by K252a. Values are mean $\pm$ SEM and at each point $4 \leq n \geq 6$. Corresponding basal release of FSH was between 3.3–4.6 ng/ml and was unaffected by any of the inhibitors or their vehicles at the concentrations used.

TABLE 1

EFFECT OF STAUROSPORINE ON LHRH-INDUCED LH RELEASE OVER THE DAYS OF THE ESTROUS CYCLE AND IN MALES

Hemipituitaries were incubated in vitro for 3 consecutive hours. In the basal hour either medium alone (controls) or staurosporine (300 nM) was present. In the following 2 h there was, in addition, LHRH (0.85 nM). Values given are the mean ± SEM.

Days of cycle	Inhibitor	LH (ng/ml)			*n*
		Basal hour	1st hour	2nd hour	
Estrus	Control	8.4 ± 1.6	14.2 ± 1.7	35.9 ± 2.3	4
	Staurosporine	6.2 ± 0.7	9.5 ± 1.1	9.2 ± 1.1 **	4
Metestrus	Control	4.9 ± 1.2	5.3 ± 0.4	25.1 ± 2.2	4
	Staurosporine	6.1 ± 1.4	4.6 ± 1.0	5.8 ± 1.3 **	4
Diestrus	Control	5.25 ± 1.17	8.63 ± 0.99	55.70 ± 4.94	6
	Staurosporine	2.92 ± 0.56	4.63 ± 0.37	6.15 ± 0.56 **	6
Proestrus	Control	5.25 ± 0.67	27.03 ± 2.59	159.7 ± 14.43	6
	Staurosporine	6.58 ± 0.78	25.4 ± 3.03	30.70 ± 4.26 **	6
Males	Control	6.10 ± 1.40	10.15 ± 1.77	21.03 ± 4.85	4
	Staurosporine	8.04 ± 1.04	8.70 ± 0.69	5.74 ± 0.45 *	4

* $p < 0.05$ significantly different compared to control.
** $p < 0.01$ significantly different compared to control.

TABLE 2

EFFECTS OF SOME PROTEIN KINASE INHIBITORS ON LHRH-INDUCED LH AND FSH RELEASE AND PRIMING

Hemipituitaries were incubated in vitro for 3 h. In the basal hour there was medium alone (control) or a protein kinase inhibitor. In hours 1 and 2 there was, in addition, LHRH (0.85 nM). Values given are mean ± SEM.

Inhibitor	LH (ng/ml)			*n*
	Basal hour	1st hour	2nd hour	
Control	20.7 ± 2.8	81.9 ± 8.3	285.1 ± 21.2	8
Genistein (50 μM)	14.0 ± 3.3	79.0 ± 14.8	336.3 ± 35.2	4
Control	8.5 ± 1.8	36.2 ± 8.2	191.7 ± 26.7	6
5-Iodo-C8 (10 μM)	15.6 ± 3.0	92.0 ± 11.7 **	282.5 ± 20.2 *	6
Control	7.7 ± 1.7	123.4 ± 13.5	283.0 ± 6.0	5
48/80 (30 μg/ml)	10.6 ± 2.6	184.7 ± 10.9 *	299.5 ± 11.2	5
Control	4.2 ± 0.6	37.7 ± 5.0	134.3 ± 12.0	5
Calmidazolium (1 μM)	5.8 ± 1.5	71.0 ± 11.8 *	215.6 ± 22.4 *	6
Control	7.9 ± 3.1	46.9 ± 6.3	159.0 ± 12.7	4
ML-7 (3 μM)	4.2 ± 0.5	57.0 ± 5.4	212.7 ± 26.8	4
	FSH (ng/ml)			
	Basal hour	1st hour	2nd hour	
Control	5.01 ± 0.29	9.93 ± 0.96	25.47 ± 3.52	8
Genistein	4.29 ± 0.44	7.75 ± 0.97	22.59 ± 3.97	4
Control	3.55 ± 0.38	5.89 ± 0.64	17.76 ± 2.29	4
5-Iodo-C8	5.08 ± 0.34	10.27 ± 0.82 *	28.57 ± 3.28	6
Control	2.63 ± 0.28	10.11 ± 2.38	41.72 ± 1.23	5
48/80	4.18 ± 0.47	18.16 ± 0.94 *	45.83 ± 3.62	5
Control	2.76 ± 0.23	7.50 ± 1.15	17.85 ± 2.09	4
ML-7	2.68 ± 0.19	8.49 ± 0.59	25.09 ± 2.29 *	4

* $p < 0.05$ significantly different compared to control.
** $p < 0.01$ significantly different compared to control.

1974b and Table 1). At each stage of the cycle, staurosporine (300 nM) blocked LHRH-induced gonadotropin release in the 2nd h but only to a level equivalent to that seen in the 1st h of LHRH incubation.

The less active congener of staurosporine K252a (30 nM–10 μM) also caused a concentration-dependent inhibition of the 2nd h of LHRH-induced gonadotropin release (Figs. 1*b* and 2*b*) again without affecting initial gonadotropin release. There was a significant decrease ($p < 0.05$) in LHRH-induced release of both LH and FSH compared to control at K252a concentrations of 3 μM and above. The IC_{50} for inhibition of LHRH-induced release by K252a was 2.9 ± 0.4 μM for LH and was 2.3 ± 0.5 μM for FSH.

Figs. 1*c* and 2*c* show the effect on LHRH priming of a PKC inhibitor of quite different structure, H7 (10–300 μM). At concentrations above 30 μM H7 there was significant ($p < 0.05$) inhibition of both LH and FSH release in the 2nd h with LHRH, with no effect on LHRH-induced gonadotropin release in the 1st h. The IC_{50} for inhibition of the 2nd h of LHRH-induced release by H7 was 71.6 ± 13.3 μM for LH and 88.1 ± 20.4 μM for FSH. The novel and highly-selective PKC inhibitor, Ro 31-8220 (0.1–50 μM), also caused a concentration-dependent inhibition of the primed but not the unprimed gonadotropin secretion induced by LHRH (Fig. 1*d*). At concentrations of 10 μM and above, the inhibition was statistically significant ($p < 0.05$) and gave an IC_{50} for inhibition of the 2nd h of LHRH-induced LH release of 19.0 ± 6.8 μM.

A range of inhibitors of different classes of kinases (used at concentrations described in the literature to cause more than 50% inhibition of the relevant kinase in whole cells) were compared (Table 2). Genistein (50 μM), an inhibitor of several tyrosine kinases (Akiyama et al., 1987), had no effect on either initial LHRH-induced gonadotropin release or priming. Calmidazolium (R24571), 48/80 (Gietzen, 1983) and 5-iodo-C8 (MacNeil et al., 1988), which inhibit Ca^{2+}/calmodulin-dependent kinases, all significantly increased LHRH-induced release of LH and FSH in either the 1st, 2nd or both hours with LHRH, but did not block the priming effect. Similarly, ML-7 which is a selective inhibitor of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) acting at a site other than the calmodulin recognition site (Saitoh et al., 1987), failed to block LHRH priming.

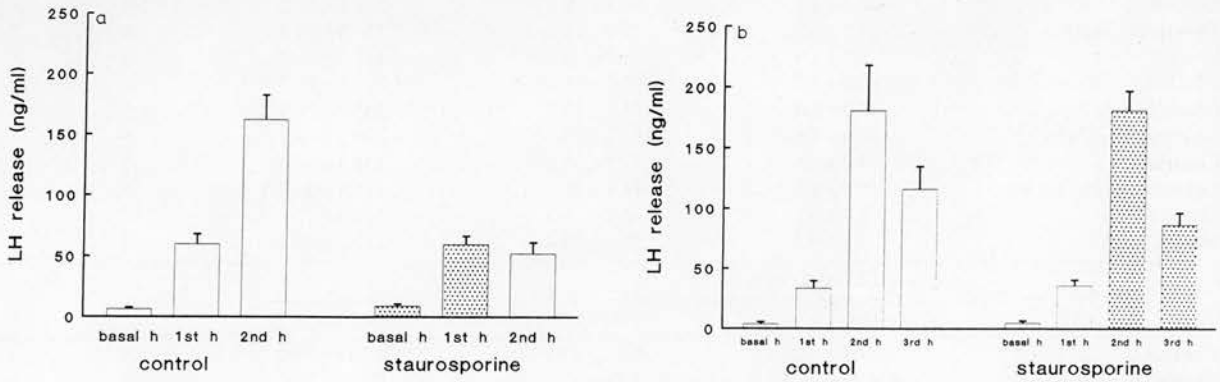

Fig. 3. Effect of staurosporine on the induction/expression of LHRH priming. Release of LH from proestrous hemipituitaries was measured over either 2 or 3 consecutive hours with LHRH (0.85 nM). In part *a*, staurosporine (300 nM) was included (dotted bars) in the basal h and 1st h of LHRH (i.e. that hour during which priming takes place). Between the 1st and 2nd h with LHRH, the tissue was washed twice with MEM to remove the staurosporine before incubating the tissue with LHRH alone for a 2nd h. Although staurosporine was not present in the 2nd h, primed release was significantly inhibited ($p < 0.05$). Part *b* shows the effect of staurosporine (300 nM) on LHRH-stimulated LH release from previously-primed proestrous tissue. Hemipituitaries were primed by incubating with LHRH for 1 h. They then received a further 2 consecutive hours of incubation in the presence of LHRH and 300 nM staurosporine (dotted bars). Staurosporine was unable to inhibit LHRH-stimulated LH release from previously-primed tissue. Values are mean $\pm$ SEM ($n = 4$).

In order to determine whether the staurosporine-sensitive step was in the induction of the priming effect rather than its expression, several experiments were carried out. Firstly, staurosporine (300 nM) was included in the basal and 1st h of LHRH incubation (i.e. that hour during which priming takes place), after which staurosporine was removed and the tissue was extensively washed before a second hourly incubation in the presence of LHRH only (see Fig. 3*a*). As observed previously, staurosporine did not alter initial LHRH-induced LH release. However, LHRH-induced release of LH in the 2nd h of LHRH was still fully blocked to a level equivalent to that in the 1st h responses to LHRH. Two pieces of evidence indicated that this washing procedure was successful in substantially reducing the staurosporine concentration in the tissue: (i) an identical procedure using [^{3}H]*N*,*N*-dimethyl staurosporine (a close structural analogue of staurosporine) resulted in a reduction of the tissue concentration to $8 \pm 2\%$ ($n = 4$) of the initial level; (ii) in tissue incubated with staurosporine and washed as above, the facilitation of K^+-induced $^{45}Ca^{2+}$ influx caused by 300 nM PDBu (MacEwan and Mitchell, 1991) was not reduced from that in control tissue ($108 \pm 10\%$ of the facilitation in vehicle-incubated controls, $n =$ 3). In the second series of experiments we investigated whether staurosporine affected LHRH-induced LH release from previously-primed tissue (Fig. 3*b*). In this experiment, tissue was primed with LHRH for 1 h before including staurosporine (300 nM) during the 2nd and 3rd h LHRH incubations. Even over a period of 2 h (to allow for the slow development of any possible effect) staurosporine did not block LHRH-induced gonadotropin release from previously-primed tissue. The results of these two experiments show clearly that staurosporine blocks the priming but not the gonadotropin-releasing action of LHRH. The time course of the action of staurosporine is shown in Table 3. When staurosporine was added over four 30 min incubation periods with LHRH, the increment in LH release due to priming seen in periods 2, 3 and 4 of control (LHRH alone) incubations was abolished completely. However, when staurosporine was added later, at the beginning of periods 2 or 3, primed release was progressively less inhibited. The ability of LHRH priming to escape inhibition by staurosporine if the tissue is exposed to LHRH for 30 min prior to the inhibitor is in agreement with data using the protein synthesis inhibitor cycloheximide, which also prevents LHRH priming with similar temporal characteristics (Picker-

TABLE 3

EFFECT OF STAUROSPORINE ON THE TIME COURSE OF LHRH-INDUCED LH RELEASE

Release of LH was measured from hemipituitaries that were incubated in vitro for a basal 30 min and then four consecutive 30 min periods with LHRH (0.85 nM). The basal release of LH was stripped from the values for LHRH-induced LH release in the time periods 1–4. The PKC inhibitor, staurosporine (300 nM) was added into the incubation series at the beginning of the time period at which + is indicated in the row above the data and was present to the end of the experiment. Values given are mean ± SEM ($n = 4$).

	LH (ng/ml)			
	30 min incubations			
	1	2	3	4
Staurosporine	–	–	–	–
LHRH	17.6 ± 10.5	52.7 ± 14.5	104.6 ± 10.2	137.2 ± 16.4
Staurosporine	–	–	+	+
LHRH	6.8 ± 3.4	35.8 ± 2.9	84.9 ± 8.6	76.5 ± 3.9 *
Staurosporine	–	+	+	+
LHRH	8.2 ± 3.7	30.7 ± 11.4	57.2 ± 17.1	62.9 ± 20.4 **
Staurosporine	+	+	+	+
LHRH	17.3 ± 7.0	25.5 ± 9.8	20.8 ± 8.3 **	20.5 ± 8.4 **

* $p < 0.05$ significantly different compared to control (LHRH alone).
** $p < 0.01$ significantly different compared to control (LHRH alone).

ing and Fink, 1976). Some priming still occurred if pituitaries were exposed to LHRH for as little as 15 min before a concentration of cycloheximide was added that was sufficient to abolish priming if given in conjunction with LHRH.

To confirm that staurosporine has no inhibitory activity upon the secretory apparatus of the cell, we investigated the effect of this drug on ionomycin (50 μM)-induced release of LH and FSH. In the presence of 300 nM staurosporine, there was clearly no significant alteration in LH or FSH release induced by a 1st h or 2nd h incubation with ionomycin (Table 4). In the final experiment hemipituitaries were primed with LHRH (0.85 nM) either in the presence or absence of staurosporine (300 nM) and ionomycin (30 μM) was used as a receptor-independent secretagogue to elicit primed LH release. After priming with LHRH, ionomycin induced a much greater release of LH (157 ± 11 ng/ml) than in control incubations with ionomycin alone (63 ± 6 ng/ml). The addition of staurosporine during the priming hour, however, fully prevented this facilitation of the response to ionomycin (LH release of 69 ± 4 ng/ml; $n = 4$–6).

Discussion

The results presented here indicate that activation of a protein kinase, probably a form of PKC, is necessary for LHRH priming but not initial LHRH-induced gonadotropin release. Thus, the facilitation of gonadotropin release brought about by LHRH priming was completely prevented by staurosporine, leaving LHRH-induced release equivalent to that of unprimed responses, regardless of the degree of priming that had occurred (Table 1). The absolute potency of staurosporine here (26.3 ± 7.0 nM) is similar to that reported in thoroughly-characterised PKC-mediated cellular responses, for example, in platelets, T cells and spinal afferent nerve terminals (Davis et al., 1989; Dunn and Rang, 1990) and in cell-free assays of the activity of partially- or extensively-purified PKCs (Nakadate et al., 1988; Schaap and Parker, 1990; Johnson and Mitchell, unpublished). Indeed, under equivalent conditions to the present experiments, the release of LH elicited by 100 nM PDBu was similarly inhibited by staurosporine with a IC_{50} of 44.0 ± 17.2 nM ($n = 4$). The potency ratio for the inhibitory effects of staurosporine and K252a on LHRH priming (62- and 111-fold for FSH and LH respectively) is of a similar order to that reported for their inhibition of PKC in cell-free systems (36-fold) but contrasts with the corresponding values for cAMP-dependent (PKA) and cGMP-dependent (PKG) protein kinases of 2.5 and 2.3 respectively (Kase et al., 1986; Tamaoki et al., 1986). A novel PKC inhibitor, Ro 31-8220 (which is reported to show selectivity for PKC over PKA and Ca^{2+}/calmodulin-dependent kinase of 2–3 orders of magnitude; Davis et al., 1989), also inhibited LHRH priming of LH release, with an IC_{50} value of 19.0 ± 6.8 μM, similar to that for inhibition of PDBu (300 nM)-induced LH release (15.6 ± 4.5 μM, $n = 5$). The isoquinoline inhibitor of PKC, H7, also inhibited LHRH priming. There was, however, an interesting disparity between the IC_{50} of H7 on LHRH priming (71.6 ± 13.3 μM) and on PDBu (100 nM)-induced LH release (1.7 ± 1.5 μM, $n = 6$). We have previously described several other instances where phorbol ester-induced effects were inhibited with unusually low potency of H7 but not of staurosporine (Johnson and Mitchell, 1989; Johnson et al., 1989; Fink et al., 1990; MacEwan and Mitchell, 1991; Thomson et al., 1991a) and the ability of staurosporine but not H7 to inhibit some phorbol ester effects has also been described by others (Watson et al., 1988; Nakadate et al., 1989). Indeed when the PDBu-induced kinase activity of PKCs partially purified from anterior pituitary but not brain tissue was measured in a mixed micelle assay (Johnson et al., 1991), a Ca^{2+}-independent component of the activity was apparent which displayed a reduced potency towards H7 but not staurosporine. Both Ca^{2+}-dependent and Ca^{2+}-independent PDBu-induced activity were inhibited by staurosporine with similar IC_{50} values (114 ± 65 nM and 107 ± 41 nM respectively), but H7 inhibited Ca^{2+}-dependent activity (9 ± 4 μM) much more effectively than Ca^{2+}-independent activity (47 ± 20 μM). In brain and a number of peripheral tissues, staurosporine and H7 both displayed identical IC_{50} values on Ca^{2+}-dependent and -independent activity.

Since both staurosporine and H7 are considered to act close to the ATP-binding site of PKCs, which is quite highly conserved in a number of kinases, such disparities in the potency of H7 but not staurosporine are at first sight unexpected. However, the sites of action of these inhibitors appear to be non-identical. While K252a has been reported to be a competitive inhibitor with respect to ATP (Kase et al., 1987) there is evidence to the contrary for staurosporine (Tamaoki et al., 1986; Rüegg and Burgess, 1989) and the binding of [^{3}H]*N*,*N*-dimethyl staurosporine is displaced with only extremely low potency by H7 ($IC_{50} > 500$ μM) (Thomson et al., 1991b). Although kinase inhibitors of the isoquinoline type do appear to act in a kinetically competitive manner with respect to ATP (Hidaka et al., 1984), certain analogues (for example HA 1004 and H7) demonstrate distinct relative selectivity for or against particular kinases (Hidaka and Hagiwara, 1987) indicating that a degree of recognition specificity is possible at this site. Furthermore, it is not clear that the H7 recognition site is entirely concurrent with that for ATP, since H7 provides only partial protection against denaturation of the ATP site by covalent chemical reagents (Ohta et al., 1988). Finally, in some cases, the relationship between H7 and ATP recognition may be complex since the sequences of α and β isoforms of PKC contain a second consensus recognition motif for ATP (Huang, 1989). It is unclear whether the relatively H7-resistant kinase involved in LHRH priming might represent a particular PKC isozyme, some modified form of PKC, a distinct but related kinase or the influence of other as yet quite unknown factors. Whichever is the case, the consensus of inhibition of LHRH priming by four PKC inhibitors, taken with the ineffectiveness of an array of inhibitors of other types of kinase, strongly suggests that a form of PKC or related kinase is required for LHRH priming.

Staurosporine and K252a can have inhibitory actions on tyrosine kinases (Yamaguchi and Kathuria, 1988), PKA (Tamaoki et al., 1986), myosin light chain kinase and Ca^{2+}/calmodulin-dependent (CaM II) kinase (Watson et al., 1988). However, the action of staurosporine on LHRH priming seems unlikely to involve these kinases since inhibitors of tyrosine kinase (genistein), CaM II kinase (the calmodulin antagonists: calmidazolium, 5-iodo-C8 and 48/80) and MLCK (ML-7) were ineffective and both the potency ratio of staurosporine versus K252a on LHRH priming and the identical potency of Ro 31-8220 on priming and PDBu-induced responses argue against a role of cyclic nucleotide-dependent kinases. Interestingly, both initial LHRH-induced release and/or that from primed tissue were enhanced by a number of the CaM II kinase inhibitors. Evidence supporting a role of CaM II kinase in LHRH-induced gonadotropin release has been provided by several laboratories (Conn et al., 1984; Das et al., 1989) but recent evidence (Van der Merwe et al., 1990b) suggests that it is only the more non-specific of the calmodulin antagonists used that inhibit LHRH-induced LH release. Certainly raised intracellular Ca^{2+} is an effective secretagogue for LH, yet it will not mimic the priming effect of LHRH (Table 4). Interestingly, removal of extracellular Ca^{2+} during an initial hour of incubation with LHRH, while reducing LH release in that hour, does not prevent LHRH priming if Ca^{2+} is replaced in a second hour with LHRH (Pickering and Fink, 1979). If CaM II kinase is involved in the first phase of LHRH-induced release it is unclear why our results show an increase of LH release in the

TABLE 4

EFFECT OF STAUROSPORINE ON IONOMYCIN-INDUCED LH AND FSH RELEASE

Hemipituitaries were incubated in vitro for 3 h. In the basal hour there was medium alone (controls) or staurosporine (300 nM). In the next 2 consecutive hours there was, in addition, ionomycin (50 μM). Values given are the mean ± SEM ($n = 7$).

Inhibitor	LH (ng/ml)		
	Basal hour	1st hour	2nd hour
Control	5.5 ± 1.0	95.5 ± 13.8	63.1 ± 12.8
Staurosporine (300 nM)	6.1 ± 0.4	108.6 ± 13.0	61.9 ± 8.7
	FSH (ng/ml)		
	Basal hour	1st hour	2nd hour
Control	2.47 ± 0.16	8.65 ± 0.90	6.52 ± 0.67
Staurosporine (300 nM)	2.45 ± 0.14	7.66 ± 0.63	5.27 ± 0.38

presence of CaM II kinase inhibitors. The Ca^{2+}/calmodulin-dependent phosphatase, calcineurin (Armstrong, 1989) has been detected in pituitary cells (Farber et al., 1987), so if raised Ca^{2+} levels in gonadotropes were to increase both calmodulin-dependent phosphorylations and dephosphorylations then interpretation of the overall effects of calmodulin antagonists would be complex.

Staurosporine exerts its inhibitory effects on primed gonadotropin release if present during that hour in which LHRH priming takes place (i.e. the 1st h), but is removed for the hour of further challenge with LHRH, showing that its action is on the induction rather than the expression of LHRH priming (Fig. 3*a*). Furthermore, the response of previously-primed tissue to LHRH was unaltered by staurosporine (Fig. 3*b*). Since staurosporine will also prevent the facilitated response to ionomycin seen in previously-primed tissue, staurosporine cannot simply be acting as a slowly-acting inhibitor of a late component of LHRH responses. Additional evidence that staurosporine was not acting by a delayed, non-specific action on the secretory apparatus of the gonadotrope was provided by its lack of effect alone on secretion induced by the Ca^{2+} ionophore, ionomycin (Table 3). In an analogous fashion to its role here in LHRH priming, PKC has been implicated in the induction but not expression of another phenomenon of increased cellular responsiveness, long-term potentiation (LTP) of synaptic transmission in hippocampus (Malinow et al., 1989). The induction of LTP can be prevented by selective peptide inhibitors of CaM II kinase as well as by PKC inhibitors (Malinow et al., 1989), but it is not yet possible to say with any certainty whether such factors, in addition to PKC, serve any role in LHRH priming.

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Modulation by phorbol 12,13-dibutyrate of dihydropyridine-sensitive hormone release from rat anterior pituitary tissue *in vitro*

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Our previous studies revealed quite different influences of activators of protein kinase C (PKC) on hormone secretion from gonadotrophes and somatotrophes (Johnson & Mitchell, 1989; Johnson *et al.* 1989*b*). Phorbol 12,13-dibutyrate (PDBu)-induced release of growth hormone (GH), unlike that of luteinizing hormone (LH), occurs rapidly and is sensitive to the L-type Ca^{2+} channel blocker nimodipine (NMD). It is clear from experiments measuring $^{45}Ca^{2+}$ movements that L-channels in pituitary cells can be directly modulated by PKC (Johnson *et al.* 1989*a*). In order to investigate further the influence of PKC on L-channels in pituitary cells we examined phorbol ester and dihydropyridine effects on K^+-induced release of GH and LH.

Female COB/Wistar rats, that had been maintained under controlled lighting and temperature were killed at 13.30 h of pro-oestrus and their anterior pituitary glands removed and hemisected. The secretion of LH and GH *in vitro* was measured as described previously (Johnson *et al.* 1989*b*). Incubation with 60 mM-K^+ medium for 1 h caused a 6·7-fold increase in GH release over basal and a 9·2-fold increase in LH release. In the presence of 1 μM-NMD these responses were 97% and 87% inhibited respectively. In the presence of 100 nM-PDBu, K^+-induced GH release was unaltered, showing a mean increment of 5733 ± 1236 ng GH/ml compared to 5438 ± 531 ng GH/ml in controls (mean $\pm$ S.E.M., $n = 5$). In contrast K^+-induced LH release was amplified almost 2-fold by PDBu, showing a mean increment of $174{\cdot}6 \pm 10{\cdot}8$ ng LH/ml compared to $90{\cdot}5 \pm 11{\cdot}0$ ng LH/ml in controls. The K^+-induced release of GH in the presence of PDBu was reduced by 5355 ± 718 ng/ml by 1 μM-NMD whereas the corresponding LH response was only reduced by $97{\cdot}3 \pm 8{\cdot}7$ ng/ml (an amount similar to the K^+-induced release without PDBu amplification). However, when PDBu-induced hormone release was studied in the presence of 3 μM-BAY K8644 (which had no effect alone during 1 h of incubation), the LH response was greatly potentiated with an increment of 2·56-fold over that of PDBu-induced LH release alone. The release of GH was completely unaffected. It is possible that in gonadotrophes but not in somatotrophes, L-type Ca^{2+} channels are recruited by PDBu in a form (Hess *et al.* 1984) which requires the additional influence of another factor such as BAY K8644 before optimal activity is expressed.

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D 124 T

THE ACTIVATION OF PROTEIN KINASE C ISOFORMS BY 1,2-DIOCTANOYL-*sn*-GLYCEROL (DOG) COMPARED WITH PHORBOL 12,13-DIBUTYRATE (PDBu). M.S. Johnson, R. Mitchell, D.J. MacEwan and F.J. Thomson, MRC Brain Metabolism Unit, 1 George Square, Edinburgh, UK.

To investigate the possibility that phorbol esters and diacylglycerols may display some selectivity in the activation of protein kinase C (PKC), we used a mixed-micelle histone IIIS kinase assay to determine PKC activity. Phosphatidyl serine-dependent kinase activity partially-purified from cytosol was measured in the presence (100 μM) or absence (< 3 nM) of free Ca^{2+}. EC_{50} values were determined for PDBu and DOG using tissue sources rich in particular isoforms; i.e. midbrain (most isoforms), spleen (ß, with some δ) and COS 7 cells (α). With Ca^{2+} present, PDBu activated PKC from all three sources similarly, with EC_{50} s (nM):77 ± 10 (spleen), 148 ± 73 (COS 7) and 104 ± 128 (midbrain). The corresponding values without Ca^{2+}, were 620 ± 117, 2270 ± 1595 and 875 ± 131. When DOG was used as activator the corresponding EC_{50}s (μM) were 17.3 ± 11.7, 19.0 ± 5.4 and 2.1 ± 0.4, in the presence of Ca^{2+}. Without Ca^{2+}, the EC_{50} values were 84.5 ± 23.0, no activation (COS 7) and 41.3 ± 14.7 respectively. The total PKC activity evoked without Ca^{2+} was greater for PDBu than DOG, but was similar with Ca^{2+} present, suggesting that PDBu, (but not DOG, at αPKC at least) can activate some Ca^{2+}-dependent PKC isoforms in a Ca^{2+}-independent fashion. However, DOG is more effective at activating a Ca^{2+}-dependent PKC isoform in midbrain that is not α or ß and thus may well be γ.

D 173 T

PHARMACOLOGICALLY DISTINCT FORMS OF PROTEIN KINASE C REGULATE ARACHIDONIC ACID RELEASE FROM ANTERIOR PITUITARY CELLS. F.J. Thomson, D.J. MacEwan, M.S. Johnson & R. Mitchell, MRC Brain Metabolism Unit, 1 George Square, Edinburgh, UK.

Activation of protein kinase C (PKC) can induce arachidonic acid (AA) release from anterior pituitary cells. Since different forms of PKC may display selective pharmacology, we investigated the actions of activators and inhibitors of PKC on [^{3}H]AA release from prelabelled rat anterior pituitary pieces. [^{3}H]Arachidonic acid release from anterior pituitary tissue was significantly increased in the presence of either phorbol 12,13-dibutyrate (PDBu), mezerein or 1,2-dioctanoyl sn-glycerol. The [^{3}H]AA release induced by PKC activators was readily blocked by staurosporine and Ro 31-8220. However, blockade by another PKC inhibitor, H7, was biphasic, consisting of H7-sensitive and insensitive components. Furthermore, concentrations of H7 as high as 100 μM could only block PDBu-induced AA release to a level 23 ± 9 % above basal. PKC activity, partially-purified from anterior pituitary cytosol, was measured in a mixed micelle assay using histone IIIS as a substrate. Phosphatidylserine-dependent, PDBu-induced, Ca^{2+}-independent activity was relatively resistant to block by H7 (IC_{50} = 121 ± 18 μM) in comparison to Ca^{2+}-dependent activity (IC_{50} = 17 ± 4 μM). Corresponding values for staurosporine were 101 ± 30 nM and 171 ± 46 nM, respectively. Clearly both H7-resistant, Ca^{2+}-independent and H7-sensitive, Ca^{2+}-dependent PKCs (or related kinases) can participate in the control of AA release.

Q 102 F

INVERSE REGULATION OF L-TYPE Ca^{2+} CHANNELS BY TWO DISTINCT FORMS OF PROTEIN KINASE C. R. Mitchell, D.J. MacEwan, M.S. Johnson, F.J. Thomson, MRC Brain Metabolism Unit, 1 George Square, Edinburgh, UK.

In order to investigate kinase regulation of Ca^{2+} channels in pituitary cells we measured effects of kinase activators and inhibitors on the depolarisation-induced rapid influx of $^{45}Ca^{2+}$. Phorbol esters such as phorbol 12,13-dibutyrate (PDBu) elicited inverse effects on nimodipine-sensitive K^+-induced $^{45}Ca^{2+}$ influx in GH_3 cells (inhibition) and anterior pituitary prisms (facilitation). Both influences were stereospecific and readily blocked by staurosporine (3 - 1000 nM) and also by Ro 31-8220. H7 showed clear selectivity between the responses, with an IC_{50} in GH_3 cells of 7 ± 4 μM but in pituitary prisms of 62 ± 6 μM. 1,2 Dioctanoyl-*sn*-glycerol and a number of atypical phorbol esters could elicit the facilitatory but not the inhibitory effect, whereas mezerein caused similar facilitation in both tissues. The effect of mezerein in GH_3 cells was blocked by staurosporine, Ro 31-8220 and H7 with IC_{50}s of 23 ± 9 nM, 7 ± 4 μM and 59 ± 8 μM. This suggests that mezerein may be a selective activator of one or more form of PKC (which is relatively resistant to H7) and can select a distinct facilitatory component out of the combination of inhibition and facilitation elicited by a broad-spectrum activator such as PDBu.

Differential actions of protein kinase C modulators on the release of hormones from rat anterior pituitary tissue *in vitro*

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We have previously reported that phorbol 12,13-dibutyrate (PDBu)-induced release of luteinizing hormone (LH) and growth hormone (GH) from rat anterior pituitary *in vitro* show different time courses and different sensitivity to the protein kinase C (PKC) inhibitors H7 and staurosporine (Johnson & Mitchell, 1989). In view of the possibility that distinct forms of PKC may be involved, we have examined the effects of another PKC activator, mezerein (MEZ), known to mimic only some of the effects of phorbol esters (Slaga *et al.* 1980).

The release of LH and GH from pro-oestrous rat hemipituitaries was measured according to Johnson & Mitchell (1989). The magnitude of GH release induced by MEZ was similar to that seen with PDBu, thus in the presence of either 100 nM PDBu or 100 nM MEZ, GH release was 2.32 ± 0.33 or 2.33 ± 0.33 (mean ± S.E.M., $n = 4$–6) fold of basal. However, MEZ (100 nM) was more effective at releasing LH, where the equivalent figures were 3.52 ± 0.28 and 13.35 ± 1.92 fold of basal LH release respectively ($n = 4$–6). The release of GH induced by MEZ or by PDBu (300 nM) was unaffected by H7 (30 μM). In contrast, 10 μM H7 inhibited the release of LH induced by MEZ and PDBu by 35 ± 10 and $64 \pm 15\%$ respectively ($n = 4$–6).

To compare the actions of these PKC modulators on kinase activity, partially purified PKCs from male rat midbrain (reported to contain messenger RNA for all of the known PKC isoforms; Scott Young III, 1989) were investigated using a phosphatidyl serine-dependent, histone III-S kinase assay, similar to that described by Huang *et al.* (1988). The Ca^{2+}-independent activity induced by MEZ and PDBu was inhibited similarly by H7 (IC_{50}s of 27 ± 7 and 49 ± 4 μM, $n = 4$–8). The Ca^{2+}-dependent activity induced by MEZ was notably more sensitive to H7 than that induced by PDBu (IC_{50}s of 4 ± 2 and 47 ± 9 μM, $n = 3$–8). Taken together, these data suggest that MEZ may show some selectivity for activation of a Ca^{2+}-dependent PKC, which is highly sensitive to H7. Both this and other PKCs may participate in the LH release induced by PKC activators.

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PHARMACOLOGY OF PROTEIN KINASE C ISOFORMS AND THEIR CELLULAR ACTIONS IN ANTERIOR PITUITARY CELLS.

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The multiple isoforms of protein kinase C (PKC) may have a range of distinct functions. We are interested in whether they can be differentially manipulated pharmacologically in anterior pituitary cells. A number of distinct pharmacological profiles emerge from experiments on PKC regulation of secretion and ion fluxes in pituitary cells. Arachidonic acid (AA), like phorbol dibutyrate (PDBu),acts through PKC in GH_3 cells to attenuate $^{45}Ca^{2+}$ influx through 'L'-type Ca^{2+} channels. However, in anterior pituitary tissue, AA, cannot mimic the PDBu-induced enhancement of $^{45}Ca^{2+}$ influx through 'L'-channels. Likewise, in pituitary tissue, PDBu-induced secretion of luteinizing hormone (LH), but not growth hormone (GH), is mimicked by AA. These PKC-mediated effects are all sensitive to the PKC inhibitor staurosporine. However, only the influences of PKC on GH_3 (but not pituitary) Ca^{2+} influx and LH (but not GH) secretion were H7-sensitive. Selectivity of dioctanoyl glycerol (DOG) and deoxyphorbol isobutyrate (DPB) will also be discussed. These activators and inhibitors of PKC may be selective for PKC-isoforms in functional pituitary cells.

Protein kinase C may be required to maintain activity of nimodipine-sensitive Ca^{2+} channels in rat anterior pituitary cells *in vitro*

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Potassium-induced release of growth hormone (GH) from anterior pituitary tissue *in vitro* is inhibited by nimodipine (Johnson *et al.* 1991). Correspondingly, K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and into cells of the GH_3 line is nimodipine-sensitive (Johnson *et al.* 1991; Johnson *et al.* 1989). The protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu) also releases GH and the response is largely inhibited by nimodipine (97 ± 9% inhibition at 1 μM, mean ± S.E.M., $n = 6$), suggesting that a PKC in somatotrophes can enhance the activity of dihydropyridine-sensitive Ca^{2+} channels. The K^+-induced influx of $^{45}Ca^{2+}$ in anterior pituitary prisms is facilitated by PDBu and both this response and the effect of PDBu on GH release were acutely reversed by the PKC inhibitor staurosporine (3–300 nM) but not H7 (1–30 μM) (Johnson *et al.* 1989; Johnson & Mitchell, 1989). The present experiments were carried out to investigate whether there is a tonic influence of PKC on these Ca^{2+} channels.

Measurements of GH secretion and K^+-induced $^{45}Ca^{2+}$ influx were made as previously described (Johnson & Mitchell, 1989; MacEwan & Mitchell, 1990). Staurosporine (300 nM) reduced 60 mM K^+-induced GH release during consecutive hours to 44 ± 13 and 10 ± 14% of controls after 1 h addition: preincubation with the drug (mean ± S.E.M., $n = 8$). There was no effect of 10 μM H7 (114 ± 15% of control; mean ± S.E.M., $n = 5$), nor did staurosporine reduce ionomycin (50 μM)-induced release (107 ± 14 and 100 ± 12% of control in 2 consecutive hours, mean ± S.E.M., $n = 7$). Correspondingly, several selective PKC inhibitors reduced K^+-induced $^{45}Ca^{2+}$ influx into pituitary prisms after preincubation for 60 min but not 10 min: for example, 300 nM staurosporine caused 84 ± 7 and 7 ± 5% inhibition at the two time points respectively (mean ± S.E.M., $n = 4$). Ionomycin (30 μM)-induced $^{45}Ca^{2+}$ influx was unaffected by staurosporine (300 nM for 60 min) and K^+-induced $^{45}Ca^{2+}$ influx was slightly enhanced by H7 to 138 ± 10% of control by 20 μM H7 for 60 min (mean ± S.E.M., $n = 4$). Furthermore, presumed down-regulation of PKC level in GH_3 cells by treatment with PDBu (300 nM) for 24 h reduced 60 mM K^+-induced $^{45}Ca^{2+}$ influx to 29 ± 10% of control levels (mean ± S.E.M., $n = 6$).

These results support the hypothesis that PKC activity in somatotrophs may normally contribute to maintaining nimodipine-sensitive Ca^{2+} channels in a relatively activated state.

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Regional differences in the affinity of diacylglycerol analogues for [^{3}H]-phorbol 12,13-dibutyrate binding sites

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Diacylglycerols are one of the endogenous activators of protein kinase C (PKC) (Nishizuka, 1988). We have previously postulated that the short chain diacylglycerol, 1,2-dioctanoyl *sn*-glycerol (DOG) may selectively activate an isoform(s) of PKC (Johnson *et al,* 1989). Since phorbol esters, such as phorbol 12,13-dibutyrate (PDBu), are known to activate PKC by acting at the diacylglycerol binding site, we investigated the effect of a range of 1,2-diglycerides on cytosolic [^{3}H]-PDBu binding from various regions in the rat with known contents of the different Ca^{2+}-dependent PKC-isoforms.

Cytosolic [^{3}H]-PDBu binding was performed as previously described (MacEwan & Mitchell, 1990). The range of 1,2-diglycerides consisted of unmixed saturated chains of 6 -18 carbon atoms ($C_{6:0}$ - $C_{18:0}$), 1,2-dioleoyl *sn*-glycerol (DO) ($C_{18:1}$, cis-9) which has two unmixed chains with one unsaturated double bond in each, and a mixed unsaturated chain diacylglycerol, 1-stearoyl-2-arachidonoyl *sn*-glycerol (SAG) ($C_{18:0}/C_{20:4}$,[cis,cis,cis,cis]-5,8,11,14). When measuring the IC_{50} for reversal of specific binding by 0 - 500 μM of each compound in rat lung, frontal cerebral cortex and cerebellum, DO and SAG showed the highest affinities, which were similar in each of the three regions tested (11 - 23 μM with DO, and 6, 4 and 4 μM with SAG for lung, cortex and cerebellum respectively (n = 4)). However, the IC_{50} values for $C_{6:0}$ - $C_{18:0}$ saturated diglycerides varied considerably, with their affinities in lung being consistently lower than in the other regions for all the chain lengths tested. Differences in affinity were not due to selective actions of lipases because DOG pre-incubated in lung had the same subsequent affinity for binding in cortex as DOG pre-incubated in cortex or cerebellum. Plots of affinity against chain length were bell-shaped and showed lowest affinity for [^{3}H]-PDBu-binding with $C_{6:0}$ and $C_{18:0}$ (IC_{50}s were 50 - 176 μM for $C_{6:0}$, $C_{8:0}$ and $C_{18:0}$ in cortex and cerebellum and 1120, 1354 and 416 μM in lung respectively). The highest affinity in the saturated diglyceride series was with a C_{14} chain length (IC_{50}s of 17 μM for cortex and cerebellum; 46 μM in lung). Using cytosol from various tissues with known contents of Ca^{2+}-dependent PKC-isoforms (Shearman *et al,* 1988), the affinity of DOG varied not only using lung, cortex and cerebellum, but with all the regions tested and an inverse relationship was apparent between the affinity for [^{3}H]-PDBu binding and the proportion of α-PKC isoform present in the tissue.

There is clearly an optimal chain length and composition for diacylglycerol interaction with [^{3}H]-PDBu binding sites, but it now appears that short chain saturated diglycerides such as DOG may selectively interact with certain isoforms of PKC.

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198 SELECTIVITY OF PROTEIN KINASE C ACTIVATORS IN THE REGULATION OF Ca^{2+}-CHANNELS IN RAT ANTERIOR PITUITARY CELLS

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Protein kinase C (PKC) can be activated not only by diacylglycerols and phorbol esters but also by arachidonic acid (AA) which can selectively activate certain PKC-isoforms. Phorbol 12,13-dibutyrate (PDBu) has previously been shown to enhance depolarisation-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary pieces, but inhibits $^{45}Ca^{2+}$ influx into GH_3 clonal cells under similar conditions[1]. This influx occurs through an L-type Ca^{2+}-channel in both pituitary pieces and GH_3 cells. Amongst a range of PKC activators tested, on K^+-induced $^{45}Ca^{2+}$ influx in these models, 1,2-dioctanoyl glycerol (DOG), 12-deoxyphorbol 13-isobutyrate (DPB) and AA could selectively elicit just one of the responses. K^+-induced $^{45}Ca^{2+}$ influx into pituitary pieces was increased by 108 ± 10% in the presence of 100μM DOG and by 30 ± 5% in the presence of 1μM DPB. DOG and DPB were inactive in GH_3 cells. In contrast, AA inhibited K^+-induced $^{45}Ca^{2+}$-influx into GH_3 cells (IC_{50} = 20μM) through activation of PKC[2] but was inactive in pituitary pieces up to 300μM. There is evidence that both α- and β- but not γ- isoforms of PKC are present in the pituitary[3]. It is possible that the selective actions of certain PKC activators here may be due to their preferential activation of particular PKC isoforms.

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Heterogeneous profiles of protein kinase C activation

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Depolarisation-induced calcium influx in anterior pituitary prisms and in GH_3 cells are both mediated by a nimodipine-sensitive process and are modulated in two ways by activation of protein kinase C (PKC) (Johnson *et al.,* 1989). Phorbol 12,13-dibutyrate (PDBu) activation of PKC leads to an increase in K^+-induced $^{45}Ca^{2+}$ influx in anterior pituitary pieces, but a decrease in GH_3 cells. These actions are also seen with 4ß-phorbol 12,13-didecanoate (4ß-PDD) but not 4α-PDD. The two opposing profiles may represent the actions of different forms of PKC on 'L'-channels in the two preparations. A number of diterpenes have been described which differentially display certain aspects of phorbol-like actions, for example, inflammation/tumour promotion/ornithine decarboxylase induction (Dunn & Blumberg, 1983). The present experiments investigate the actions of a range of such analogues in the Ca^{2+} influx models.

$^{45}Ca^{2+}$ influx measurements into anterior pituitary prisms and GH_3 cells were as previously described (MacEwan and Mitchell, 1990). In pituitary prisms, the marked facilitation of influx caused by (10-1000nM) PDBu and 4ß-PDD (~+180% at 1000nM) was mimicked, but to a lesser degree, by 1,2-dioctanoyl-sn-glycerol (1-100µM) (DOG), mezerein, phorbol 12-retinoate 13-acetate (PRA), phorbol 12-myristate 13-acetate 4-0-methyl ether (MPMA) and to a small degree by 12-deoxyphorbol 13-isobutyrate (DPB) (all at 10-1000nM). In GH_3 cells, PDBu and 4ß-PDD reduced K^+-induced $^{45}Ca^{2+}$ influx by up to 50% and a similar effect, of greater magnitude, was displayed by arachidonic acid (AA) (MacEwan and Mitchell, 1990). In contrast, DOG, PRA, MPMA and DPB were inactive. Surprisingly, the effect of mezerein in GH_3 cells was a facilitation of K^+-induced $^{45}Ca^{2+}$ influx, apparently identical to that in pituitary prisms. The effects of diterpenes were not altered by differing times of pre-incubation nor by VO_4^{3-} (1mM) or substitution of Na^+ by N-methyl-d-glucamine. The results are consistent with the idea that at least two forms of PKC can exert qualitatively different influences over 'L'-type Ca^{2+} channel function. The two complex profiles could be explained if different relative amounts of two distinct forms of PKC were involved in the two models. Whilst PDBu and 4ß-PDD were non-selective activators, mezerein and AA may be selective activators of the PKC forms that facilitate or attenuate $^{45}Ca^{2+}$ influx respectively. The other analogues may show partial selectively for the facilitatory effect. Although other explanations of these profiles may well be possible, selective antagonism by H7, but not staurosporine, of the effect of PDBu in GH_3 cells but not in pituitary prisms (Johnson *et.al.,* 1989) supports our hypothesis.

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Protein kinase C-dependent and -independent actions of arachidonic acid in GH_3 cells

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The influx of $^{45}Ca^{2+}$ evoked by exposure of GH_3 cells to high K^+-containing medium is mainly (~85%) through a nimodipine-sensitive route [1]. Pre-exposure of GH_3 cells to arachidonic acid (AA) causes a time- and concentration-dependent inhibition of K^+-induced $^{45}Ca^{2+}$ influx which is reversed by selective inhibitors of protein kinase C (PKC) [2] and attenuated by down-regulation of PKC levels by 24 h pre-treatment with phorbol-12,13-dibutyrate (PDBu). The inhibition of K^+-induced $^{45}Ca^{2+}$-influx by AA and other unsaturated fatty acids occurred in the folowing order of magnitude at 30 μM: AA > linoleic acid > AA methyl ester, which agrees with their order of potency in activation of PKC [3]. A similar inhibition of K^+-induced $^{45}Ca^{2+}$ influx was seen with 10 min PDBu treatment prior to the depolarising stimulus [2]. Either PDBu or 4ß (but not α) –phorbol 12,13-didecanoate, however, caused a maximal inhibition of depolarisation-induced $^{45}Ca^{2+}$ influx of ~50%, whereas AA fully inhibited K^+-induced $^{45}Ca^{2+}$ influx at a concentration of [illegible] μM. A selective inhibitor of PKC, H7 [4], fully reversed the inhibition seen with either PDBu or AA, but HA1004, a less potent and unselective kinase inhibitor, could not reverse the effect of PDBu or AA. The same distinction was apparent with the PKC-selective and -unselective kinase inhibitors staurosporine [5] and K252a [6] respectively. Therefore, the involvement of PKC in AA-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells seems likely, but an additional (non PDBu-like) action of AA must account for the full inhibition of $^{45}Ca^{2+}$-influx seen with AA and not PDBu.

Fluorimetric studies to determine the cytosolic calcium concentrations within GH_3 cell populations were performed using the fluorescent dye Indo-1 [7]. Briefly, GH_3 cells were loaded with 10 μM Indo-1 acetoxy methyl ester (Indo-1-AM) for 45 min in culture medium (Hams F-10, 15% foetal bovine serum, 1 mM l-glutamine, 100 u/ml penicillin and 0.1 mg/ml streptomycin) at 37°C, 5% CO_2, 95% air. Cells were harvested, washed and resuspended in Hank's Balanced Salt Solution (HBSS (Gibco, Paisley, U.K.)). A 30 min incubation in the dark at room temperature was allowed to provide more complete hydrolysis of intracellular Indo-1-AM. Cells were washed by resuspension-centrifugation (1,000 g, 10 min, 25°C) and again resuspended in HBSS to a density of 5 x 10^6 cells/ml. A sample (2.5 ml) was aliquoted to a quartz cuvette (37°C, stirred magnetically) and the fluorescence was measured in a Shimadzu RF-5000 spectrofluorophotometer interfaced with an IBM PS/2 55 SX micro-computer. Excitation (332 nm, band width 5 nm) and emission (400 nm, band width 5 nm) wavelengths were kept constant so as to reduce the possibility of significant auto fluorescence artefacts in the final signal [8] and a data point was taken every 2 s.

AA (17 μM) induced a rise in GH_3 cytosolic calcium concentrations which was unaffected by the cyclo-oxygenase and lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 30 μM) and to H7 (30 μM). Higher concentrations of AA could not be tested because of vehicle and solubility artefacts. No rise in cytosolic Ca^{2+} level was apparent with 300 nM PDBu. If a 40 mM K^+ challenge was applied to the cells 5 min after addition of AA, then the K^+-stimulated rise in cytosolic Ca^{2+} concentration was greatly diminished, concurring with the $^{45}Ca^{2+}$ influx data. In the additional presence of H7 (30 μM), the K^+-induced response was fully restored. Therefore, the initial AA-induced rise in cytosolic calcium was insensitive to H7, whereas, the inhibition by AA of K^+-induced elevation of cytosolic Ca^{2+} levels was prevented by H7.

In $^{45}Ca^{2+}$ efflux studies, where the prelabelled cells were exposed to a 50 μM AA challenge, there was a marked increase in the rate of $^{45}Ca^{2+}$ release from the cells. This increase in Ca^{2+} efflux seen with AA was not mimicked by PDBu (300 nM) nor blocked by H7 (30 μM) or cyclo-oxygenase and lipoxygenase inhibitors (indomethacin (10 μM), eicosatetraynoic acid (10 μM) or NDGA (10 μM)). The increased $^{45}Ca^{2+}$ efflux due to AA was blocked by the Ca^{2+} ATPase inhibitor Na_3VO_4 (1 mM) but was unaffected by replacing Na^+ in the medium with N-methyl d-glucamine (in order to inhibit the Na^+/Ca^{2+} exchanger).

The inhibition of depolarisation-induced $^{45}Ca^{2+}$ influx by AA seems to be mediated by PKC activation just like the PDBu-induced inhibition of Ca^{2+}-influx [1]. Unlike PDBu, AA can cause a rise in cytosolic calcium concentrations which is not due to metabolism of AA, but is presumably due to AA itself releasing intracellularly stored Ca^{2+}, as seen in other cellular systems [9-11]. It seems likely that it is this AA-induced rise in cytosolic calcium that underlies the activity of AA in the $^{45}Ca^{2+}$ efflux studies. Thus the AA-stimulated rise in Ca^{2+} efflux may simply reflect the calcium handling processes of GH_3 cells whereby raised cytosolic calcium is extruded from the cells via a Ca^{2+}-ATPase, but not a Na^+/Ca^{2+} exchange mechanism. Both the fluorimetric and $^{45}Ca^{2+}$ efflux studies have shown that this AA-induced rise in cytosolic calcium is unaffected by cyclo-oxygenase or lipoxygenase inhibitors and is not mimicked by PDBu. Our theory, therefore, is that the additional inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx in GH_3 cells when comparing AA to PDBu action, is due to the additional ability of AA to release intracellularly stored Ca^{2+}. This raised cytosolic calcium concentration could act to potentiate the activation of PKC by AA and lead to a full inhibition of nimodipine-sensitive Ca^{2+} entry. Other Ca^{2+}-stimulated mechanisms of diminishing the K^+-stimulated $^{45}Ca^{2+}$ entry apart from PKC cannot be ruled out, although the inhibition of Ca^{2+} entry caused by both AA and PDBu are fully H7- and staurosporine-reversible (but not fully reversed with their inactive congeners, HA1004 and K252a respectively, at the same concentrations).

AA can be metabolised to a wide range of cellular mediators which may be involved in the action of exogenously applied AA in GH_3 anterior pituitary cells, but in the present study have remained uninvestigated. It is likely that AA-induced release of intracellularly stored Ca^{2+} may have a necessary role in the enhanced PKC-mediated inhibition of 'L'-type Ca^{2+} channel activity seen with AA rather than phorbol esters. A dual intracellular calcium release and PKC activating response may be required to see the full inhibition of 'L'-type Ca^{2+} channel activity. Such synergistic dual actions of AA may result in an ability of AA to act as a far more important physiological activator of PKC than has been previously recognised.

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Inhibition of depolarisation-induced calcium influx into GH_3 cells by arachidonic acid: the involvement of protein kinase C

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The influx of $^{45}Ca^{2+}$ induced in GH_3 cells by exposure to 60 mM K^+ medium was inhibited by arachidonic acid (AA) in a concentration-dependent manner. This action of AA was not prevented by inhibitors of its metabolism but was reversed by the inhibitors of protein kinase C (PKC), H7 and staurosporine but not their less active congeners HA 1004 and K252a, respectively. Presumed down-regulation of PKC by pretreatment with phorbol 12,13-dibutyrate (PDBu) also greatly diminished the effect of AA. Experiments to assess effects of AA on $^{45}Ca^{2+}$ efflux and on cytosolic Ca^{2+} concentrations indicated that an additional PKC-independent action of AA involving the release of intracellularly stored calcium was present. Both direct activation of certain PKC isoform(s) by AA and the synergistic influence on PKC activity by its concomitant raising of intracellular Ca^{2+} concentrations, may be physiologically important in the regulation of depolarisation-induced Ca^{2+} entry.

Introduction

Arachidonic acid (AA) can be metabolised to a range of bioactive compounds by means of cyclo-oxygenase, lipoxygenase and other pathways. A number of these metabolites appear to be involved in inflammatory responses, and may also have roles in neural excitation and contraction of smooth muscle [1]. For example, in *Aplysia* sensory neurons, where the neurotransmitter FMRFamide is known to generate AA [2], an unknown AA-metabolite was indicated in having a role in synaptic transmission [3]. The leukotriene, LTC_4 and/or a related metabolite has been implicated in the opening of potassium channels [4,5] in guinea-pig and rat heart.

It is increasingly clear that AA production occurs when a number of transmitters act on their receptors; for example the M1 and M3 muscarinic [6], NMDA [7] and $GABA_B$, bradykinin, α_1-adrenergic and vasoactive intestinal peptide [8] receptors all stimulate production of free AA in receptive cells. Whether this is secondary to phospholipase C activation is unclear, but a functional interaction of G-protein $\beta\gamma$-subunits with phospholipase A_2 has been described in bovine retina rod outer segments [9]. Thus, it seems quite possible that either AA itself or its metabolic products may have an important role in cellular signalling by certain neurotransmitters.

It may be that AA itself also has a significant role in signalling processes. Arachidonic acid has been shown to release calcium from hepatocyte microsomes [10,11]. However, the relationship between the AA-sensitive pool and the inositol 1,4,5-trisphosphate (IP_3)-sensitive pool is not entirely clear [12]. It has further been shown that AA can inhibit IP_3 production in pancreatic cells [13] perhaps through an ATP-depleting process, and evoke IP_3 accumulation in astrocytes [14] an effect which may be due to a raised intracellular calcium level. Both AA and its metabolite 12-hydroperoxyeicosatetraenoic acid (12-HPETE) have also been described to markedly inhibit type II calcium/calmodulin-dependent kinase in rat forebrain synapto-

Abbreviations: PKC, protein kinase C; AA, arachidonic acid; IP_3, inositol 1,4,5-trisphosphate; NDGA, nordihydroguaiaretic acid; ETYA, eicosa-5,8,11,14-tetraynoic acid; 8-Br-cyclic GMP, 8-bromoguanosine-3′:5′-cyclic monophosphate; *N*-methyl TRH, *N*-methylthyrotrophin releasing hormone; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochlorine; HA 1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; DTPA, diethylenetriamine-pentaacetic acid; Indo-1-AM, indo-1-acetoxymethylester.

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somes [15]. Transport processes such as glutamate uptake into glial cells may also be influenced by AA [16] possibly by increasing membrane fluidity.

A further putative target for AA and its metabolites is protein kinase C (PKC); of which at least seven distinct isoforms have been identified so far [17]. Nishizuka and his co-workers have shown that AA itself can selectively activate the γ-and α-isoforms of PKC in cell-free systems [18]. Little is known however, of whether such events may be of physiological importance.

In anterior pituitary cells, AA has been reported to increase the secretion rates of several hormones. Luteinizing hormone and follicle stimulating hormone secretion were enhanced in response to exposure to AA [19]. Prolactin secretion [20] and adrenocorticotrophic hormone secretion [21] are also increased by an AA stimulus.

In the present experiments we investigated some of the cellular influences of AA on the GH_3 clonal anterior pituitary cell line; in particular the effects on depolarisation induced Ca^{2+} influx. In view of the quantitative differences observed in the influences of AA and other PKC activators on depolarisation-induced $^{45}Ca^{2+}$ influx, we carried out further studies (Ca^{2+} fluorimetry; $^{45}Ca^{2+}$ efflux) to clarify any additional action of AA.

Materials and Methods

Materials

GH_3 cells and foetal bovine serum were obtained from Flow Laboratories, Irvine, Strathclyde, U.K. Ham's F-10 medium and Hanks' balanced salt solution were supplied by Gibco-BRL, Paisley, Strathclyde, U.K. Penicillin, streptomycin, L-glutamine, essential fatty acid-free bovine serum albumin, arachidonic acid (sodium salt), arachidonic acid-methyl ester, linoleic acid, phorbol 12,13-dibutyrate, NDGA, 8-bromo cyclic GMP (sodium salt), *N*-methyl-D-glucamine, sodium orthovanadate, indomethacin and Indo-1-AM were all purchased from the Sigma, Poole, Dorset, U.K. Piperonyl butoxide was supplied by the Aldrich, Gillingham, Dorset, U.K. SKF-525A ('Proadifen') was obtained from Research Biochemicals, Natick, MA, U.S.A. *N*-Methyl TRH was purchased from Peninsula Laboratories, St. Helens, Merseyside, U.K. H7 and HA 1004 were obtained from Seikagaku America, St. Petersburg, FL, U.S.A. Staurosporine and K252a were supplied by Kyowa Medex Co., Tokyo, Japan. Ionomycin was bought from Novabiochem (U.K.), Nottingham, U.K. ETYA was a gift from Roche Products, Welwyn Garden City, U.K. and $^{45}Ca^{2+}$ (specific activity, 17 mCi/mg) was purchased from Amersham International, Amersham, U.K. All other chemicals were of Analar grade.

Methods

Cell culture. GH_3 cells were grown in Ham's F-10 medium supplemented with 15% foetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Cells were harvested by agitation, washed by resuspension centrifugation ($1000 \times g$, 10 min, 25°C) and finally resuspended at the appropriate density in the required medium.

Calcium influx studies. Cells were diluted to a density of $5 \cdot 10^6$ cells/ml in 'calcium uptake medium' (concentrations in mM: NaCl, 154/KCl, 5.4/$CaCl_2$, 1.5/D-glucose, 11/Hepes, 6 (pH adjusted to 7.4 with Tris-base) and with 0.05% fatty acid-free bovine serum albumin). Aliquots of this suspension (0.5 ml/tube) were preincubated (30 min, 37°C, O_2 atmosphere) before a 10 min incubation (37°C, O_2) with drugs or solvent alone. Cells were then exposed to 1 ml of calcium uptake medium containing either low K^+ (5.4 mM final concentration) or high K^+ (60 mM) with 4 μM $^{45}CaCl_2$ (≈ 3 μCi/tube). After 30 s (37 °C), $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of ice-cold 2 mM EGTA (Ca^{2+}-free) calcium uptake medium and tissue was separated by vacuum-filtration through Millipore SCWP cellulose acetate/nitrate filters (8 μm pore size) underlain by GF/B filters on Millipore 1225 sampling manifolds (Millipore, Harrow, U.K.). Samples were washed once immediately with 3 ml ice-cold EGTA calcium uptake medium and then washed a further three times for 2 min each in the same medium. The radioactivity associated with the cellulose filters and cells was determined by liquid scintillation counting. Preliminary experiments on GH_3 cells and extensive similar studies on anterior pituitary prisms [22] revealed that these conditions gave the optimal signal-to-noise ratio. The stimulus-induced influx of $^{45}Ca^{2+}$ in excess of basal controls was maximal within 30 s, suggesting that it represented specific response-triggered flux rather than adsorption or steady accumulation by storage pools. Vehicle (ethanol up to 0.4%) was always included in controls as appropriate and was independently determined to have no effect on either basal or K^+-induced $^{45}Ca^{2+}$ accummulation.

Calcium efflux studies. Harvested GH_3 cells were resuspended at a concentration of $1 \cdot 10^7$ cells/ml in calcium uptake medium containing 0.05% essential fatty acid-free bovine serum albumin and incubated for 20 min (37°C, O_2) before addition of 1 ml of medium containing 6 μM $^{45}Ca^{2+}$ (≈ 4.5 μCi/tube). Samples were incubated for 1 h at 37°C under O_2 before loading onto separate GF/B filters pre-washed with calcium uptake medium and under vacuum on a Millipore 1225 sampling manifold. Cells were then washed three times for 2 min with 3 ml of pre-warmed (37°C) calcium uptake medium alone. There then followed a further eight washes with pre-warmed calcium uptake

medium containing the appropriate inhibitor or solvent for controls. Each successive 2 min wash with 3 ml of calcium uptake medium (±inhibitor) was collected. The 4th and subsequent washes contained 50 μM AA. Radioactivity in each fraction was measured by liquid scintillation counting.

Cytosolic calcium measurements. Cytosolic Ca^{2+} concentrations were measured by use of the calcium fluorescent dye Indo-1 [23]. GH_3 cells were loaded with 10 μM Indo-1-AM for 45 min in culture medium at 37°C, 5% CO_2/95% air. Cells were then harvested, washed and resuspended in Hanks' balanced salt solution. A 30 min incubation in the dark at room temperature was then given to allow more complete hydrolysis of intracellular Indo-1-AM. The suspension was then centrifuged ($1000 \times g$, 10 min, 25°C) and the cell pellet was again resuspended in Hanks' balanced salt solution at a concentration of $5 \cdot 10^6$ cells/ml. A sample (2.5 ml) of the cell suspension was loaded into a quartz cuvette which was stirred magnetically and maintained at a constant temperature of 37°C. Fluorescence was measured in a Shimadzu RF-5000 spectrofluorophotometer. Excitation was at 332 nm (band width = 5 nm) and emission was at 400 nm (band width = 5 nm). Emission wavelengths were not ratioed between 400 and 490 nm so that the possibility of introducing significant autofluorescence artefacts in the final signal [24] would be reduced. A fluorescence value was taken every 2 s and at the end of each experiment, 10 μM ionomycin followed by 10 mM $MnCl_2$ (final concentrations) were added to give a measure of the maximum and minimum fluorescent values of the cuvette contents, respectively. An assessment of extracellular fluorescence resulting from dye leakage was made according to the method proposed by Rink and Pozzan [25]. Thus, appropriate corrections were made for the small changes in basal fluorescence induced by the addition of 100 μM $MnCl_2$ which were rapidly and fully reversed by 200 μM DTPA. The experimental values for cytosolic Ca^{2+} concentrations were calculated according to Grynkiewicz et al. [23] and Luckhoff [24]. In view of the limited solubility of AA in aqueous media, we carried out experiments to assess light-scattering using excitation and emission wavelengths of 520 nM and maximum sensitivity of the fluorimeter. Under our conditions (calcium uptake buffer containing 0.4% ethanol vehicle) there was no detectable increase in light-scattering up to and including 32 μM AA. At 38 μM AA, irreversible turbidity was detectable and by 44 μM this was very marked. All cell Ca^{2+} fluorescence experiments were thus carried out with AA at concentrations below the threshold for turbidity (Fig. 6).

Data analysis. Statistical significance of effects was assessed by a non-parametric method (Mann-Whitney *U*-test). Concentration-response curves were analysed by a non-linear iterative curve-fitting program ('P-fit'; Biosoft, Cambridge, U.K.). The calculated concentration which inhibits 50% of the maximal response (IC_{50}) is expressed, with the errors representing standard errors of the mean.

Results

Depolarisation with 60 mM K^+ medium caused a marked influx of $^{45}Ca^{2+}$ (see Table I) which was inhibited in a time-dependent manner by 30 μM AA. The inhibitory effect of AA was apparent without any preincubation but an additional 1 min preincubation produced a maximal inhibitory effect. At concentrations up to 100 μM, AA had no effect on basal accumulation of $^{45}Ca^{2+}$ (104 ± 8% of control at 100 μM). Inhibition of K^+-induced $^{45}Ca^{2+}$ influx by AA was concentration-dependent (Fig. 1) with an IC_{50} (concentration that gives 50% inhibition of maximal response) of 19 ± 3 μM. Agents with some structural similarity to AA; AA-methyl ester (30 and 100 μM) and linoleic acid (30 μM) were unable to mimic the inhibition seen with AA (30 μM). The inhibitory effect of AA was virtually maximal by the concentration (38 μM) at which AA reached the limit of its solubility under these conditions. Higher concentrations of AA did not significantly affect basal $^{45}Ca^{2+}$ accumulation (Table I) or that induced by ionomycin or *N*-methyl TRH (Table IV) suggesting that even exceeding the solubility limit of AA had little influence on the results of $^{45}Ca^{2+}$ influx experiments. Furthermore, it was clear that AA-methyl ester (which was less soluble than AA under our conditions; showing marked light-scattering

TABLE I

Time-course of inhibition of depolarisation-induced calcium influx by arachidonic acid (AA) into GH_3 cells

Values represent means ± S.E., $n = 4$–6. The total $^{45}Ca^{2+}$ accumulated in the presence of 60 mM K^+ was typically around 1600 dpm per assay, whereas basal $^{45}Ca^{2+}$ accumulation was around 600 dpm, of which non-specific adsorbtion to the filter and cell surfaces (determined in zero-time blanks) was responsible for around 400 dpm. $^{45}Ca^{2+}$ accumulation was measured over 30 s and the incubation times with AA are inclusive of the 30 s influx measurement period. Incubations were at 37 °C and $^{45}Ca^{2+}$ influx was stopped as described under Materials and Methods. Statistically significant inhibition of K^+-induced $^{45}Ca^{2+}$ influx is indicated by (* $P < 0.05$).

Conditions	$^{45}Ca^{2+}$ accumulation (fmol/10^6 cells per min)
Non-specific adsorbtion (zero-time blank)	34 ± 2
Basal	50 ± 4
Basal, 100 μM AA (10 min)	54 ± 6
60 mM K^+	120 ± 8
60 mM K^+, 30 μM AA (30 s)	86 ± 6 *
60 mM K^+, 30 μM AA (45 s)	64 ± 3 *
60 mM K^+, 30 μM AA (90 s)	52 ± 3 *

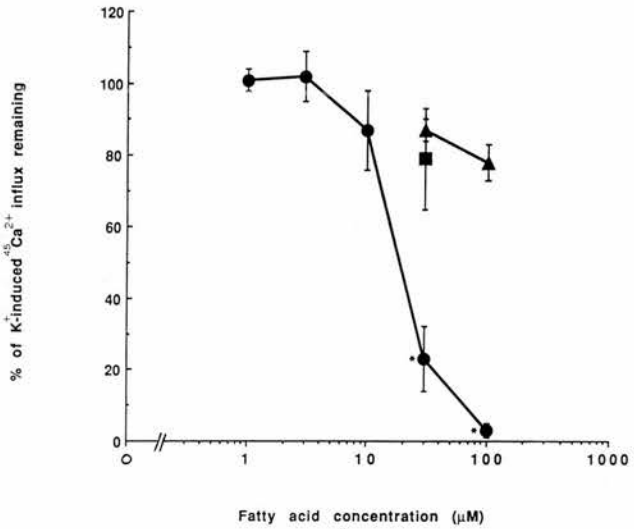

Fig. 1. Concentration-response data for the inhibition of depolarisation-induced calcium influx by fatty acids. Cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37 °C and calcium influx was stopped as described under Materials and Methods. Cells were preincubated with arachidonic acid (AA, circles), arachidonic acid-methyl ester (AA-methyl ester, triangles) and linoleic acid (square) for 10 min at 37 °C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Values represent means ± S.E., $n = 4$–12. Statistically significant inhibition of K^+-induced $^{45}Ca^{2+}$ influx is indicated by (* $P < 0.05$).

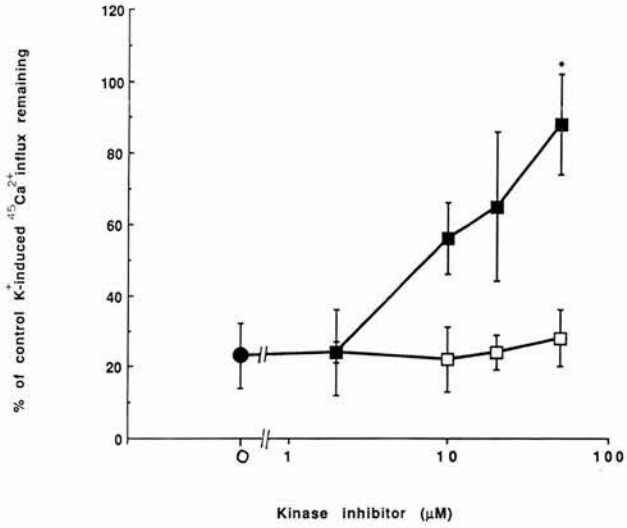

Fig. 2. Reversal by H7 but not HA1004 of the inhibition by AA of depolarisation-induced calcium influx. GH_3 cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37 °C and calcium influx was stopped as described under Materials and Methods. H7 (filled squares) and HA1004 (open squares) were added immediately prior to addition of AA (30 μM), then cells were preincubated for 10 min at 37 °C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Control K^+-induced $^{45}Ca^{2+}$ influx data in the presence of AA is shown by the circle. Values represent means ± S.E., $n = 8$–12. Both H7 and HA1004 at 50 μM had no effect alone on basal or K^+-induced $^{45}Ca^{2+}$ influx. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx due to AA was significantly reversed by 50 μM H7 ($P < 0.05$).

artefacts by 24 μM) failed to mimic the inhibition of K^+-induced $^{45}Ca^{2+}$ influx caused by AA, even when tested at concentrations of 30 and 100 μM (Fig. 1). However, 30 μM AA-methyl ester in the presence of 300 nM PDBu reduced K^+-induced $^{45}Ca^{2+}$ influx levels to 14 ± 2% of control K^+-induced influx ($n = 4$).

The inhibitory effect of 30 μM AA on K^+-induced calcium influx (a reduction to 23 ± 9% of control K^+-induced influx) was reversed by the protein kinase C inhibitor H7 (Fig. 2), with an IC_{50} of 14 ± 4 μM. A congener of H7, HA1004, with much reduced activity as a PKC inhibitor [26] was inactive at similar concentrations. Similarly, the potent PKC inhibitor staurosporine also reversed the effect of 30 μM AA on K^+-induced $^{45}Ca^{2+}$ influx (IC_{50} of 52 ± 15 nM, see Fig. 3), but its less active congener K252a [27,28] failed to cause reversal. The reversibility of the effect of AA by these means rules out any simple physicochemical actions of AA or any chemical oxidation products that may arise during the experiment. Down-regulation of PKC by treatment of GH_3 cells with 300 nM phorbol ester for 24 h results in more than 50% reduction in PKC levels [29]. Using a similar protocol for PKC down-regulation [30] by treatment of GH_3 cells with 300 nM PDBu for 24 h, reduced the ability of AA to inhibit K^+-induced $^{45}Ca^{2+}$ influx (Fig. 4).

The inhibition of K^+-induced $^{45}Ca^{2+}$ influx by 30 μM AA was not influenced by inhibitors of its metabolism (Table II). The cyclo-oxygenase and lipoxygenase inhibitor ETYA (10 μM), the lipoxygenase in-

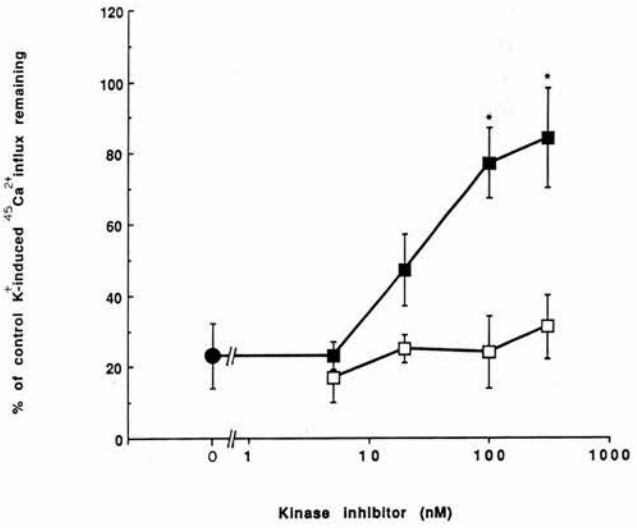

Fig. 3. Reversal by staurosporine but not K252a of the inhibition by AA of depolarisation-induced calcium influx. GH_3 cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37 °C and calcium influx was stopped as described under Materials and Methods. Staurosporine (filled squares) and K252a (open squares) were added immediately prior to addition of AA (30 μM) then cells were preincubated for 10 min at 37°C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Control K^+-induced $^{45}Ca^{2+}$ influx data in the presence of AA is shown by the circle. Values represent means ± S.E., $n = 4$–8. Both staurosporine and K252a at 300 nM had no effect alone on basal or K^+-induced $^{45}Ca^{2+}$ influx. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx due to AA was significantly reversed by 100 and 300 nM staurosporine ($P < 0.05$).

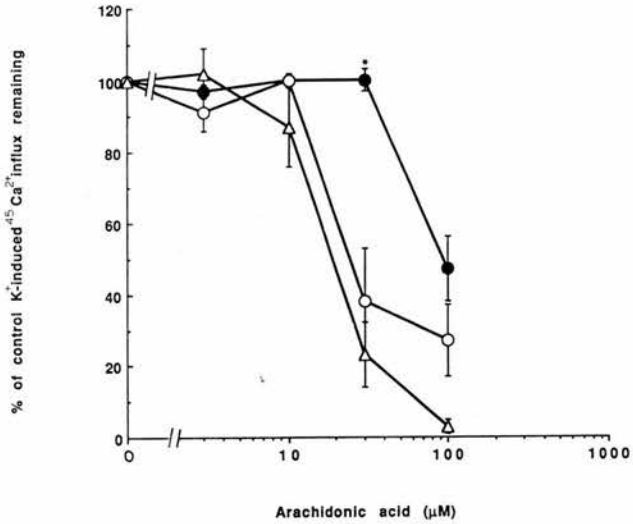

Fig. 4. Effect of PKC-down regulation on arachidonic acid inhibition of depolarisation-induced calcium influx. GH_3 cells were cultured for 24 h with 300 nM PDBu in dimethylformamide (0.01% final volume, filled circles), dimethylformamide alone (open circles) or nothing (triangles) and then extensively washed by centrifugation and resuspension in 'calcium uptake medium' (three times 10 min, 1000 $\times g$, 25°C). Arachidonic acid was added 10 min before a 30 s exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Values represent means ± S.E., $n = 6$. The reduction in $^{45}Ca^{2+}$ influx caused by AA (30 μM) was significantly attenuated (*) by prolonged preincubation with PDBu compared to its solvent alone ($P < 0.05$).

hibitor NDGA (30 μM) and the cytochrome *P*-450 inhibitors piperonyl butoxide (30 μM) and SKF 525A (10 μM) did not significantly modify the effect of AA.

Whereas 50 μM AA inhibited the influx induced by 60 mM K^+ to 3 ± 1% of control, the influx responses to 1 μM *N*-methyl TRH and 30 μM ionomycin were reduced by AA to only 77 ± 16% and 83 ± 10% of the

TABLE II

Effect of inhibitors of arachidonic acid metabolism on its inhibition of depolarisation-induced calcium influx

GH_3 cells were exposed to 30 μM alone, or in combination with 10 μM ETYA, 30 μM NDGA, 10 μM SKF 525A or 30 μM piperonyl butoxide for 10 min before and during addition of 60 mM K^+ plus $^{45}Ca^{2+}$ medium. None of these drugs caused any significant alteration in the inhibition of K^+-induced $^{45}Ca^{2+}$ accumulation due to 30 μM AA or had any apparent effect on basal K^+-induced $^{45}Ca^{2+}$ influx alone. Values represent the means ± S.E., $n = 4$–8.

Drug concentration	% of control K^+-induced $^{45}Ca^{2+}$ influx remaining
60 mM K^+ medium alone	100
+ 30 μM arachidonic acid	23 ± 9
+ 30 μM arachidonic acid + 10 μM ETYA	23 ± 4
+ 30 μM arachidonic acid + 30 μM NDGA	35 ± 8
+ 30 μM arachidonic acid + 10 μM SKF 525A	26 ± 3
+ 30 μM arachidonic acid + 30 μM piperonyl Butoxide	29 ± 5

TABLE III

Effect of inhibitors of calcium extrusion processes on ability of arachidonic acid to reduce depolarisation-induced calcium influx

GH_3 cells were harvested and then resuspended in either normal 'calcium uptake medium' with or without 1 mM Na_3VO_4 or sodium-free 'calcium uptake medium' with *N*-methyl-D-glucamine replacing sodium (154 mM). Cells were incubated with or without 100 μM arachidonic acid for 10 min before exposure to 60 mM K^+ and $^{45}Ca^{2+}$ medium. Values represent the means ± S.E., $n = 4$. Neither K^+-induced $^{45}Ca^{2+}$ nor the inhibition of that response by AA were significantly altered by Na_3VO_4 or *N*-methyl-D-glucamine.

Conditions	% of control K^+-induced $^{45}Ca^{2+}$ influx remaining
60 mM K^+	100
60 mM K^+, *N*-methyl-D-glucamine + Na_3VO_4	130 ± 8
60 mM K^+, 100 μM AA	8 ± 6
60 mM K^+, 100 μM AA, *N*-methyl-D-glucamine	30 ± 6
60 mM K^+, 100 μM AA, Na_3VO_4	16 ± 2

respective control responses (Table IV). The effect of AA on K^+-induced $^{45}Ca^{2+}$ influx therefore relates (at least mostly) to a modulation of the specific entry route involved in that response, not just a general alteration of Ca^{2+}-handling by the cell.

The cell-permeable activator of cyclic GMP-dependent protein kinase, 8-Br-cyclic GMP, was unable to mimic the effect of AA at any concentration from 3–100 μM (data not shown). Throughout this concentration range of 8-Br-cyclic GMP the maximum deviation from control K^+-induced $^{45}Ca^{2+}$ influx was 14 ± 14% (mean ± S.E., $n = 8$). Sodium orthovanadate (Na_3VO_4) and replacement of sodium with *N*-methyl-D-glucamine will effectively inhibit plasma membrane Ca^{2+}-ATPases and the Na + /Ca^{2+} exchanger, respectively [31,32]. Neither 1 mM Na_3VO_4 nor sodium replacement could prevent the inhibition of K^+-induced calcium influx caused by 100 μM AA (see Table III). The minor elevation of Ca^{2+} accumulation due to K^+ in the presence of AA that was seen with sodium orthovanadate and *N*-methyl-D-glucamine was also seen with K^+alone. It seems likely, therefore, that these small effects reflect influences of the Ca^{2+}-ATPase and the Na + /Ca^{2+} exchanger on redistribution of elevated Ca^{2+} and we could thus find no evidence that these Ca^{2+} transporters are involved in any specific way in the AA-induced inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx. Since the effect on influx of AA (Table III) was unaltered in the presence of a concentration of Na_3VO_4 which fully blocked $^{45}Ca^{2+}$ extrusion (see Fig. 5) it seems unlikely that PKC modulation of Ca^{2+}-ATPase activity [33] plays any major role in the inhibition of $^{45}Ca^{2+}$ influx observed here.

In the $^{45}Ca^{2+}$ extrusion experiments, a clear increase in the rate of $^{45}Ca^{2+}$ efflux was seen in response

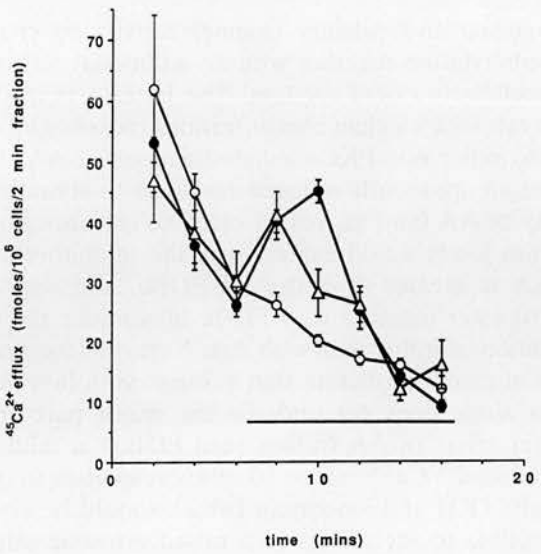

Fig. 5. Effect of arachidonic acid on calcium efflux from GH_3 cells. Measurement of $^{45}Ca^{2+}$ efflux was performed as described under Materials and Methods. Cells were batch-perfused in either the presence (triangles) or absence (filled circles) of 10 μM NDGA. Arachidonic acid (50 μM) in ethanol (or ethanol alone for control (open circles)) was added to the medium from 6–16 min where indicated by the line. Values represent the means ± S.E., $n = 3$.

to 50 μM AA (Fig. 5). This response was transient, being over within four efflux fractions, despite the continued presence of AA, suggesting that it originated from the discharge of a discrete stored pool. The effect of 50 μM AA was considerably less than (about 36% of) the efflux caused by 100 μM ionomycin. In the presence of 10 μM NDGA the response to AA appeared to be slightly attenuated and was completely prevented in the presence of 1 mM Na_3VO_4 (data omitted for clarity). There was no increase in $^{45}Ca^{2+}$ efflux rate in response to 300 nM PDBu suggesting the lack of involvement of PKC activation in this effect of AA. The $^{45}Ca^{2+}$ efflux response to AA was unaltered by 10 μM ETYA, 10 μM indomethacin, 30 μM H7 or in sodium-free medium (data not shown).

The fluorimetric studies revealed that basal cytosolic calcium concentrations were clearly elevated by quite a low concentration (17 μM) of AA alone (Fig. 6b and c), but as also reported by Drummond [33], phorbol esters were ineffective. This response to AA was not inhibited by 30 μM H7 (Fig. 6c) or 30 μM NDGA (data not shown) but quite unlike the inhibition of K^+-induced $^{45}Ca^{2+}$ influx caused by AA, was mimicked by AA-methyl ester at an equivalent concentration. The increments in calcium concentration due to AA (17 μM), PDBu (300 nM) and AA-methyl ester (17 μM) were 43 ± 11, 6 ± 8 and 52 ± 9 nM respectively (means ± S.E., $n = 3$–5). The rise in cytosolic calcium induced by 40 mM K^+ medium was markedly reduced to $14 \pm 9\%$ of control (mean ± S.E., $n = 4$) by previous addition of AA (Fig. 6b) paralleling the $^{45}Ca^{2+}$ influx data. In the presence of 30 μM H7, the rise in basal cytosolic calcium level induced by 17 μM AA was unaltered, but the inhibitory effect of AA on subsequent responses to 40 mM K^+ was markedly attenuated (Fig. 6c); the mean response to K^+ now being $89 \pm 10\%$ of control (mean ± S.E., $n = 3$). In accordance with this, presumed down-regulation of PKC in GH_3 cells by pro-

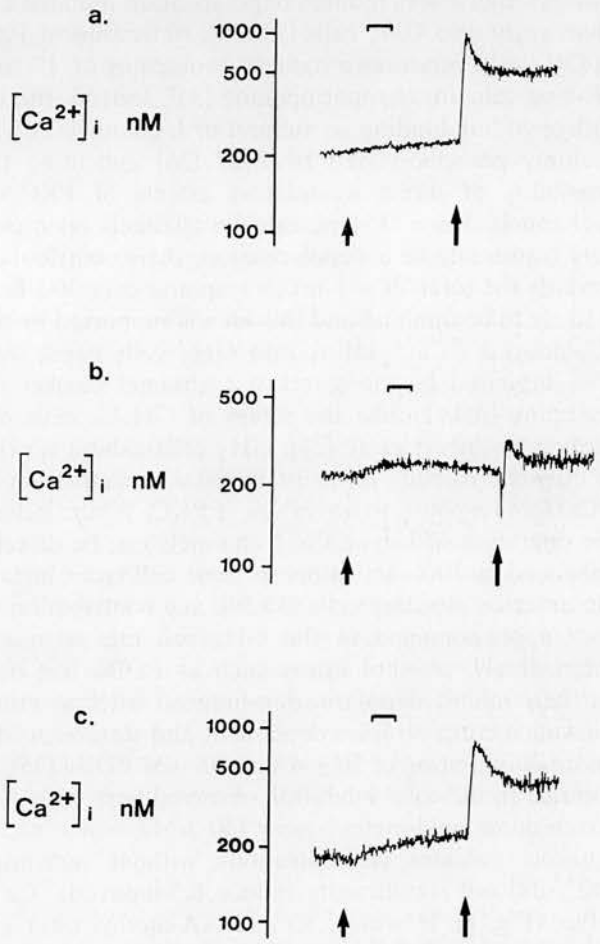

Fig. 6. Effect of arachidonic acid on the cytosolic calcium concentration in GH_3 cells. Cells were prepared and intracellular Ca^{2+} levels estimated by Indo-1 fluorescence as described under Materials and Methods. Arachidonic acid (AA, 17 μM) in ethanol (b and c) or ethanol alone (a) were added where indicated by the first (shorter) arrows. Ethanol at concentrations of up to 0.4% (as used) had no apparent effect on basal or K^+-induced increments in calcium concentrations. At the concentration used, AA produced no turbidity artefact in fluorescence records made in the absence of cells. At the second (longer) arrow, KCl was added to a final concentration of 40 mM. Addition of 40 mM NaCl rather than KCl had no effect. Part (a) illustrates a typical response to K^+ in the presence of ethanol, the vehicle for AA (0.4%). In (b), AA induced a rise in basal Ca^{2+} levels and a diminution of the subsequent response to 40 mM K^+. In (c), when 30 μM H7 had been present from the start of the record, the AA-induced rise in basal Ca^{2+} levels was still present, but the diminution by AA of the response to K^+ was reversed. There was no apparent effect of H7 alone. Staurosporine could not be tested because of the unfavourable fluoresence spectrum of the compound. Each trace is representative of at least three similar results. The abscissa represents time with each scale bar indicating 1 min.

longed preincubation with PDBu (300 nM) greatly diminished the effect of AA on the response to K^+ but not its elevation of basal calcium levels (data not shown).

Discussion

Previous work has shown that activation of PKC with phorbol esters reduces depolarisation-induced calcium entry into GH_3 cells [33–35]. Activation of PKC in GH_3 cells produces a reduced probability of 'L'- and 'T'-type calcium channel opening [34]. Indeed, the dihydropyridine-binding α_1 subunit of L-channels can be multiply phosphorylated by PKC [36] indicating the possibility of direct modulatory effects of PKC on L-channels. Since 'T'-type calcium channels open only very transiently to a depolarisation, their contribution towards the total $^{45}Ca^{2+}$ influx response over 30 s here is likely to be minimal and this idea is supported by the K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells being over 85% inhibited by the selective L-channel blocker nimodipine [35]. Unlike the strain of GH_4C_1 cells described by Albert et al. [37], GH_3 cells exhibit no rise in cytosolic calcium levels or in basal accumulation of $^{45}Ca^{2+}$ in response to activators of PKC. Whilst indeed the operation of L-type Ca^{2+} channels can be directly enhanced by PKC activators in some cell types including anterior pituitary cells [35,38], any contribution of such a phenomenon in the GH_3 cell line is minor. Interestingly, phorbol esters such as PDBu can only partially inhibit depolarisation-induced calcium influx showing a concentration-dependent and stereo-specific maximal inhibition of $50 \pm 4\%$ at 300 nM PDBu [35], in contrast to the total inhibition observed here with AA. Arachidonic acid-methyl ester (30 μM), which raises cytosolic calcium concentrations without activating PKC, did not significantly reduce K^+-induced $^{45}Ca^{2+}$ influx (Fig. 1). However, 30 μM AA-methyl ester enhanced the reduction of K^+-induced $^{45}Ca^{2+}$ influx caused by 300 nM PDBu to $86 \pm 4\%$.

Furthermore, unlike PDBu, AA also has the ability to raise cytosolic calcium concentrations in GH_3 cells. This effect is apparent in the fluorimetric studies and probably underlies the increase in $^{45}Ca^{2+}$ efflux rate induced by AA. Down-regulation of PKC in GH_3 cells leads to reduced PKC-mediated responses [29]. Although the potency of AA at inhibiting K^+-induced $^{45}Ca^{2+}$ influx was greatly reduced by presumed down-regulation of PKC, the effect was not completely prevented. This probably reflects incomplete down-regulation (as would occur with the present protocol [29]), and the contribution of an additional effect of AA, other than direct activation of PKC. In summary, it seems likely that AA may inhibit $^{45}Ca^{2+}$ influx into GH_3 cells by a mechanism with at least two components: a direct activation of PKC which may reduce voltage-sensitive calcium channel activity by channel phosphorylation together with an additional release of intracellularly-stored calcium. The latter action would raise cytosolic calcium concentrations (possibly in addition to other non-PKC-mediated actions of AA) leading to an apparently reduced response to stimuli. The ability of AA (and its methyl ester) to elevate cytosolic calcium levels would explain why the inhibitory effect of AA is greater than that of PDBu, and why AA-methyl ester together with PDBu fully mimic the total inhibition of influx seen with AA. Nevertheless, several lines of evidence dictate that raising cytosolic calcium levels alone does not underlie the major part of the greater effect of AA (rather than PDBu) in inhibiting K^+-induced $^{45}Ca^{2+}$ influx: (i) influx responses to other stimuli, TRH and ionomycin (which should be equally susceptible to the effects of a raised cytosolic calcium concentration) were almost unmodified by AA, and (ii) the whole of the effect of AA was reversed by H7 and staurosporine (but not their less active congeners) in a manner suggesting critical involvement of PKC. The simplest unifying explanation of the data would seem to be that the additional release of Ca^{2+} by AA serves to promote the efficiency with which AA can induce activation of PKC. Other explanations may of course be possible.

Arachidonic acid and some of its metabolites have been reported to open potassium channels in heart [4,5] resulting in a hyperpolarisation. As here, these effects took seconds to minutes to develop, but in contrast were prevented by lipoxygenase inhibitors. Such relatively slow development of the effect of AA here is consistent with the involvement of an enzymic mechanism such as kinase action. Similarly, maximal effects of PDBu on GH_3 cells were observed only after a preincubation of at least 1 min. These facts coupled to the selective H7 and staurosporine reversal of the AA effect (Figs. 2 and 3) support the idea that activation of PKC by AA mediates the effects observed here.

In cell-free systems, AA can selectively activate the α- and γ-isoforms of PKC [18]. Our observations indicate that material immunoreactive with polyclonal antibodies for α-, β- and ϵ-isoforms of PKC, is present in GH_3 cells (Simpson, J., MacEwan, D.J., Mitchell, R., Johnson, M.S., Thomson, F.J. and Parker, P.J., unpublished data), similar to the profile reported in the GH_4C_1 cell line where the γ-isoform has also been shown to be absent [39]. This suggests that it may be the α-isoform of PKC which mediates AA action in GH_3 cells, although any role of any further PKC isoforms in these cells cannot yet be excluded.

Linoleic acid and AA-methyl ester are less potent activators of PKC than AA [40], but do cause Ca^{2+} release from intracellular stores [10,12]. Linoleic acid and AA-methyl ester only poorly mimic AA in the calcium influx studies (see Fig. 1) but AA-methyl ester

can robustly mimic AA in the fluorimetric studies, further indicating that AA-inhibition of calcium influx cannot simply be due to release of calcium from intracellular stores.

It has been suggested [41] that lowered cytosolic calcium may result from activation by AA of guanylate cyclase and thus, activation of cyclic GMP-dependent protein kinase. This appears not to be a major factor in the AA inhibition of calcium influx seen here since 8-Br-cyclic GMP is totally without effect up to 100 μM. Arachidonic acid and certain of its metabolites can also inhibit Ca^{2+}/calmodulin-dependent protein kinase II [15], but the lack of effect of calmodulin antagonists on K^+-induced $^{45}Ca^{2+}$ influx in pituitary pieces [42] and also in GH_3 cells (Mitchell, R. and MacEwan, D.J., unpublished data) suggests that any action does not contribute significantly to the inhibition of influx caused by AA here.

The components of the response to AA in GH_3 cells may well be direct actions of AA, since inhibitors of AA metabolism are without effect (Table II and Fig. 5). Although NDGA caused a slight inhibition of AA-induced $^{45}Ca^{2+}$ efflux, any specific action was in doubt because of the lack of effect with ETYA in the same studies. The increased extrusion of $^{45}Ca^{2+}$ occuring in response to AA (Fig. 5) may be almost exclusively mediated by a Ca^{2+}-ATPase as it was completely blocked by sodium orthovanadate [31], whereas Na^+ removal was without effect [32]. Table IV shows that AA does not inhibit all stimulus-induced calcium influx to the same degree suggesting that the AA site(s) of action is at a specific route of Ca^{2+} entry rather than on calcium extrusion.

In summary, arachidonic acid inhibits depolarisation-induced calcium influx into GH_3 cells by activation of PKC (perhaps α-isoform in particular) and by raising cytosolic calcium concentrations. Both of these mechanisms seem to contribute to a reduction in calcium influx. It is likely that it is arachidonic acid itself, and not a metabolite, which mediates these effects. This appears to be the first clear evidence linking activation of PKC by AA with a physiologically-relevent consequence. Further work is required to characterise the exact PKC isoform(s) which mediate the effects of AA here and indeed what specific substrate sites this isoform might have. It seems clear that not only diacylglycerols, but also AA may be important in the physiological activation of PKC.

TABLE IV

Effect of arachidonic acid on calcium influx induced by 60 mM K^+, N-methyl TRH and ionomycin

GH_3 cell $^{45}Ca^{2+}$ influx was measured as described under Materials and Methods. Cells were preincubated with 50 μM arachidonic acid 10 min before exposure to either 60 mM K^+-, 1 μM *N*-methyl TRH- or 30 μM ionomycin-containing $^{45}Ca^{2+}$ 'calcium uptake medium' for 30 s at 37°C. Values represent the means ± S.E., $n = 4$–12. The effect of AA on the response to K^+, but not ionomycin or *N*-methyl TRH was statistically significant (* $P < 0.05$).

Stimulus	$^{45}Ca^{2+}$ accummulation (fmol/10^6 cells per min)		% Inhibition of stimulus-evoked increment in $^{45}Ca^{2+}$ influx by 50 μM AA
	− AA	+ 50 μM AA	
Nil	50 ± 10	49 ± 15	−
60 mM KCl	120 ± 18	52 ± 18	97 ± 16
1 μM *N*-methyl TRH	72 ± 6	66 ± 4	23 ± 11
30 μM ionomycin	230 ± 28	198 ± 19	17 ± 10

Acknowledgements

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Properties and [^{32}P] phosphorylation targets of a novel form of protein kinase C in pituitary

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We have described a number of cellular responses in anterior pituitary cells which are elicited by what appears to be a distinct form of protein kinase C (PKC), distinguished by its resistance to isoquinoline-type but not other PKC inhibitors [1 - 4]. Phorbol ester-induced facilitation of L-type Ca^{2+} channel function, secretion of growth hormone (GH), activation of phospholipase A_2 (PLA_2) and the endogenously-occurring self priming effect of LHRH are all readily inhibited by staurosporine and more PKC-selective indolocarbazoles but all show relatively low potency of inhibition by isoquinolines such as H7. Compared to other PKC-mediated responses examined in the same experimental conditions, the kinase in question displays diminished potency of 6 to > 20-fold for H7. In contrast, closely matched inhibitory potencies of staurosporine were seen in all our PKC response models.

The present experiments were carried out to examine the properties of this kinase at a biochemical level. Two kinds of experiments were carried out:

(1) An *in vitro* kinase activity assay using partially purified cytosolic PKCs and
(2) An analysis of PKC-mediated [^{32}P] phosphorylation of endogenous substrates in whole pituitary cells.

For the kinase activity assay, tissues were homogenised at 4°C in 0.5 ml of buffer (20 mM Tris HCl (pH 7.5) 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulphonyl fluoride) containing 0.01% leupeptin + 20 μM E-64 and centrifuged (16,000 g, 20 min, 4°C). The supernatant was collected (pellet discarded) and recentrifuged (16,000 g, 5 min, 4°C). The supernatant from the second spin was partially purified on diethylaminoethyl cellulose (DE52), prewashed with buffer containing leupeptin and E-64. After loading, columns were washed with 9 column volumes of buffer before the majority of the partially purified PKC activity was eluted with 3 column volumes of buffer containing 150 mM NaCl. Phosphatidyl serine was prepared as mixed micelles with Nonidet P-40. The assay was performed in 20 mM Tris HCl (pH 7.5) with 0.5 mM EGTA containing (final concentrations): 12.5 mM $MgCl_2$, 100 μg/ml phosphatidyl serine + 0.04% Nonidet P-40, 1.25 mg/ml histone III-S as substrate, 100 μM ATP-γ-^{35}S (7 - 20 kBq/tube) and partially purified PKCs. The PKCs were activated by 1 μM phorbol 12,13-dibutyrate (PDBu) with either 600 μM $CaCl_2$ (≡ 100 μM final free Ca^{2+}) or 5 mM EGTA (< 3nM final free Ca^{2+}). After 15 min at 30°C, reactions were stopped by addition of 20 μl of ice-cold 0.1 M ATP in 0.1 M EDTA (pH 7.0) and aliquots were spotted onto Whatman P81 paper (to bind [^{35}S] thiophosphorylated histone) and extensively washed with 75 mM H_3PO_4.

The Ca^{2+}-dependent and Ca^{2+}-independent activities from anterior pituitary showed a marked disparity in their sensitivity to H7. The IC_{50}s of Ca^{2+}-dependent and Ca^{2+}-independent kinase activity for H7 were 17 ± 4 and 121 ± 18 μM respectively, while as predicted the IC_{50}s for staurosporine were identical; 101 ± 39 and 117 ± 46 nM, respectively ($n \geq 4$). Less marked, but similar results were found in lung but not in a range of other tissues from the CNS and periphery.

For studies on endogenous phosphorylation targets, hemipituitary glands from female rats on the morning of pro-oestrus were preincubated for 30 min in 1 ml phosphate-free buffer at 37°C. After addition of 14.1 MBq ^{32}P-orthophosphoric acid for 3 h, incubations were terminated by insertion of the tissues into lysis buffer containing 9 M urea and 2% Nonidet P-40. After homogenisation, tissue samples containing equal amounts of trichloroacetic acid-precipitable ^{32}P were loaded onto isoelectric focusing gels (pH gradient approximately pH 4.8 - 7.1, linear within pH 5.3 - 6.9) and two-dimensional gel electrophoresis performed using a modified O'Farrell technique [5] with 12.5% (acrylamide, w/v) slab gels. Radiolabelled proteins were detected by direct autoradiography with Hyperfilm-'Betamax' film (Amersham). Approximately 400 spots were consistently resolved.

If present for 1 h, PDBu (300 nM, added 120 min after the ^{32}P) increased the phosphorylation of 6 pituitary proteins (approximate molecular weight/pI: 1 = 69 kDa/pI 6.1, 2 = 65 kDa/pI 7.0 - 7.1, 3 = 36 kDa/pI 6.9, 4 = 25 kDa/pI 7.0 - 7.1, 5 = 16 kDa/pI 6.2, 6 = 16 kDa/pI 6.7) compared with saline controls. Protein 6 was an abundant tissue protein and 1 - 5 were minor proteins. A seventh (minor) protein (7 = 19kDa/pI 6.0) showed increased phosphorylation only in some experiments. A 10 min incubation with phorbol (added 170 min after the ^{32}P) had a similar effect to the 1 h incubation. In experiments in which phorbol was present in all incubation media for 1 h, the PKC inhibitor staurosporine (300 nM), but not H7 (30 μM) when added 30 min prior to the phorbol, reduced phosphorylation of 11 proteins (2, 4, 5, 8 = 76 kDa/pI 7.0, 9 = 66 kDa/pI 7.0, 10 = 28 kDa/pI 7.0 - 7.1, 11 = 30 kDa/pI 6.3, 12 = 22 kDa/pI 6.6, 13 = 13 kDa/pI 6.0, 14 = 15.5 kDa/pI 5.3, 15 = 76 kDa/pI 6.9) when compared to the group treated with phorbol alone.

These results thus describe both distinct kinase activity and endogenous phosphorylation targets of an H7-resistant species of PKC (or PKC-like kinase). This kinase appears to utilise histone III-S as substrate in a Ca^{2+}-independent manner and to have a restricted tissue distribution distinct from that of any of the so far characterised PKCs.

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The effects of different classes of activators on rat midbrain protein kinase C

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It is now known that there are at least 7 distinct isoforms of protein kinase C (PKC) (Nishizuka, 1988) and messenger RNAs for all these isoforms have been detected in rat midbrain (Scott Young III, 1989). We have been interested for some time in the possibility that these isoforms may exhibit selective pharmacological properties. This idea is supported by the differential effects of PKC inhibitors on phorbol ester-induced hormone release from pituitary (Johnson & Mitchell, 1989) and the selectivity of some PKC activators on regulation of Ca^{2+} channels in these cells (MacEwan *et al,* 1990).

To directly examine the properties of the kinases, we used a mixed-micelle histone III-S kinase assay similar to that of Huang *et al* (1988). Phosphatidyl serine-dependent kinase activity from male rat brain cytosol (partially-purified on DEAE cellulose) was measured in the presence of 100 μM or zero (< 3 nM) free Ca^{2+}. Phorbol 12,13-dibutyrate (PDBu) activated Ca^{2+}-dependent PKCs with a consensus EC_{50} of 21 ± 5 nM compared to that for Ca-independent activity of 790 ± 120 nM (means ± s.e. mean, n = 8 - 20). Phorbol 12-myristate 13-acetate induced activity with similar maxima in each case and with a potency on Ca^{2+}-dependent activity around 10 fold greater than on Ca^{2+}-independent. Two compounds which showed selectivity in the modulation of Ca^{2+} channels by apparently distinct PKCs (MacEwan *et al,* 1991) were also tested. Deoxyphorbol 13-isobutyrate recruited similar maximal activity of Ca^{2+}-independent kinases (93 ± 3% of that due to PDBu), but rather less (73 ± 5%) of the maximal PDBu-evoked Ca^{2+}-dependent activity (mean ± s.e. mean, n = 8 - 9). Mezerein recruited 82 ± 5% of the maximal Ca^{2+}-independent activity evoked by PDBu but only 37 ± 5% of the Ca^{2+}-dependent increment (mean ± s.e. mean, n = 5 - 9). The diglyceride 1,2-dioctanoyl-sn-glycerol (DOG) activated the overall population of kinases with a consensus EC_{50} of 0.8 ± 0.3 μM in the presence of 100 μM Ca^{2+}. However, even at high concentrations (up to 1 mM), DOG could only elicit about half of the maximal Ca^{2+}-independent activity seen with PDBu (n = 4 - 8).

These data indicate that activity of Ca^{2+}-dependent and independent PKCs can be differentially elicited by some PKC activators. Under certain conditions, the diglyceride, (DOG) appears to be ineffective on a component of the Ca^{2+}-independent activity whereas other compounds such as mezerein may be ineffective on some of the Ca^{2+}-dependent kinases.

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183 STUDIES ON THE MECHANISM OF LHRH-INDUCED $^{45}Ca^{2+}$ INFLUX IN RAT ANTERIOR PITUITARY CELLS

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The secretion of gonadotrophins in response to LHRH is attenuated by removal of extracellular Ca^{2+}, indicating a partial requirement for Ca^{2+} influx in LHRH action. Both LHRH-induced LH secretion in vitro and the rapid influx of $^{45}Ca^{2+}$ induced by LHRH in anterior pituitary slices[1] are inhibited (the former only partially) by dihydropyridines. In bullfrog sympathetic ganglia and gonadotrophes, LHRH-induced membrane depolarisation, perhaps by inhibition of preactivated outward K^+ ('M') currents, may be the means of activation of voltage-sensitive Ca^{2+} currents. Indeed the 'M' current blocker uridine 5'-triphosphate (10-300μM) caused a concentration-dependent, nimodipine-sensitive $^{45}Ca^{2+}$ influx here. This response could not be mimicked by phorbol ester (phorbol 12-myristate 13-acetate (PMA) at concentrations below 10μM). Furthermore, the $^{45}Ca^{2+}$ influx induced by LHRH (1-100nM) was unaffected by protein kinase C inhibitors H7 and polymyxin B (10μM), and inhibited by PMA (10-300nM) or by the diacylglycerol kinase inhibitor R 59022 (10μM). Although our results suggest that LHRH-induced Ca^{2+} influx in gonadotrophes may indeed be secondary to 'M' current inhibition, they contrast with those in bullfrog sympathetic ganglia and NG 108-15 cells (but not hippocampal pyramidal cells), where PKC is suggested to mediate M current closure[2].

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PHARMACOLOGY AND CELLULAR ACTIONS OF PROTEIN KINASE C ISOFORMS (*Eur. J. Pharmacol.* **183**, 750-751)

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Protein kinase C (PKC) and phorbol ester binding sites are widely distributed and are likely to play a major role in signal transduction/cellular regulation in a number of systems. It is now clear that at least 7 isoforms of the enzyme are encoded. However, very little is known of the physiological roles of the isoforms and whether they display pharmacological differences that may permit selective intervention. We have developed a number of cellular models of PKC action allowing us to explore in a physiological context the putative selective pharmacology of different isoforms.

Experiments were carried out on anterior pituitary cells from rat or on the GH_3 cell line. It has been reported that α and β, but not γ isoforms of PKC are present in pituitary and that the pituitary-derived GH_3 contains two forms of PKC mRNA. K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells and pituitary pieces (both through L channels) are influenced quite differently by phorbol esters; the former being inhibited and the latter enhanced by 4ß but not 4α-phorbol 12, 13-didecanoate (PDD)(Johnson, MacEwan and Mitchell, 1989). While both effects were readily blocked by staurosporine, only that in GH_3 cells was readily blocked by H7 (IC_{50} = 10 μM). We sought selective agonists for the two effects, finding that arachidonic acid (AA) mimicked the effect in GH_3 cells but not pituitary (being both H7- and staurosporine-sensitive) whereas *sn*-1,2 dioctanoyl glycerol (DOG) and 12-deoxyphorbol 13-isobutyrate (DPB) were effective only in pituitary. Since AA activates α and γ isoforms but β is not considered to be present here, we suggest that the effect shown by AA is via αPKC. Ligand binding studies to cytosolic PKC showed an allosteric enhancement of [^{3}H]-PDBu binding in α-rich but not β– or γ-rich organs and CNS regions, supporting this hypothesis. In contrast, the IC_{50}s for a range of diacyl glycerols, particularly DOG, in displacing [^{3}H]-PDBu were much lower in ß-rich (or γ-rich) regions than in α-rich regions, suggesting that the facilitatory, H7-resistant profile on $^{45}Ca^{2+}$ influx in pituitary pieces was through ß-PKC.

Models at the level of hormone secretion parallel these observations (Johnson and Mitchell, 1989). PDBu-induced secretion of luteinising hormone (LH) but not growth hormone (GH) is mimicked by AA. Again the former effect is H7-sensitive but the latter resistant. Staurosporine is effective on both. The priming effect of LH-releasing hormone (LHRH) is a unique phenomenon of increased secretory responsiveness (Mitchell, Johnson, Ogier & Fink, 1988). This can be mimicked in some aspects by phorbol esters and is sensitive to staurosporine yet not H7. This profile is reminiscent of the extremely high levels of H7 required to block long-term-potentiation and we suggest that this is characteristic of the ß-isoform of PKC. It is clear that less well-characterised isoforms may also contribute to these phenomena to an as yet unknown extent.

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SELECTIVE PHARMACOLOGY OF PROTEIN KINASE C

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Activation of protein kinase C (PKC) by diglyceride (derived in part from the action of phospho-inositidase C) appears to play a major role in signal transduction/cellular regulation in many systems. More than seven isoforms of PKC are now known, which fall into two series: A; Ca^{2+} dependent and B; Ca^{2+} independent. However whilst many targets of 'PKC' have been described, very little is known of the physiological roles of particular isoforms and indeed whether they display pharmacological differences that may permit selective intervention. We have developed a number of cellular models of PKC action allowing us to explore, in a physiological context, the putative selective pharmacology of different isoforms. With the aim of assigning identities to the kinases active in particular models, we have further assessed the actions of particular drugs in ligand binding and kinase activity assays for PKC using cells and tissues enriched in particular isoforms.

One of our models for PKC(s) action is the regulation of $^{45}Ca^{2+}$ influx through L channels in the GH_3 cell line and in anterior pituitary cells. K^+- induced $^{45}Ca^{2+}$ into these different cells is influenced quite differently by phorbol esters; the former being inhibited and the latter enhanced by 4 β- but not 4α-phorbol 12, 13-didecanoate (PDD) (Johnson, MacEwan and Mitchell, 1989). While both effects were readily blocked by staurosporine, only that in GH_3 cells was readily blocked by H7 ($IC_{50} \cong 10\mu M$). We sought selective agonists for the two effects, finding that arachidonic acid (AA) mimicked the effect in GH_3 cells but not pituitary (being both H7- and staurosporine-sensitive) whereas sn-1,2 dioctanoyl glycerol (DOG) and 12-deoxyphorbol 13-isobutyrate (DPB) were effective only in pituitary. It is known that α, β, ε and not γ isoforms are present in both tissues, with GH_3 cells being rather more enriched in α. Since AA is reported to activate α and γ isoforms, but γ is not considered to be present here, we suggest that the effect shown by AA is via αPKC. In support of this hypothesis ligand binding studies to cystosolic PKC showed an allosteric enhancement of [^{3}H]-PDBu binding by AA, the magnitude of which correlates with the α content of tissue. In contrast, the affinities for a range of diacylglycerols, particularly DOG, in displacing [^{3}H]-PDBu showed if anything an inverse correlation with α content suggesting that the facilitatory, H7-resistant profile on $^{45}Ca^{2+}$ influx in pituitary pieces was through something other than αPKC.

Models at the level of hormone secretion parallel these observations (Johnson and Mitchell, 1989). PDBu-induced secretion of luteinising hormone (LH) but not growth hormone (GH) is H7-sensitive but the latter resistant, whilst staurosporine is effective on both. The priming effect of LH-releasing hormone (LHRH) is a unique phenomenon of increased secretory responsiveness (Mitchell, Johnson, Ogier & Fink, 1988). This can be mimicked in some aspects by phorbol esters and is sensitive to staurosporine yet not H7. This profile is reminiscent of the very high levels of H7 required to block long-term potential (LTP). It appears that ε as well as α, β and γ isoforms are H7-sensitive (Schaap and Parker, 1990) so some other form of PKC or related kinase may mediate the H7 resistant events observed.

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Does Inositol hexakisphosphate induce Ca^{2+} entry into GH_3 cells?

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A variety of cells have recently been described to synthesise inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) and inositol hexakisphosphate ($InsP_6$), (see [1] for review). In contrast to the lower inositol phosphates, the levels of these inositol polyphosphates do not change rapidly in response to Ca^{2+}-mobilising hormones [2]. However, marked excitatory effects were reported when microinjected into the Nucleus Tractus Solitarius of the brain stem [1], consistent with an extracellular site of action. Inositol polyphosphates are known to be produced in the GH_3 clonal pituitary cell line [2]. We have used the GH_3 cell line to investigate whether inositol polyphosphates might act extracellularly on these cells to influence their mobilisation of Ca^{2+}. The present report describes several series of experiments, begun originally in 1988, and our persistent difficulties in making an unequivocal interpretation of the data.

GH_3 cells were cultured and $^{45}Ca^{2+}$ accumulation determined as described previously [3]. After 30 s at 37°C with 2 μM $^{45}Ca^{2+}$, accumulation was quenched with cold EGTA-medium before rapid filtration and washing.

Concentration-dependent increases in $^{45}Ca^{2+}$ influx were induced by TRH, K^+ and ionomycin with peak increments over basal controls of 148 ± 23% (100 nM TRH) 262 ± 30% (60mM K^+) and 1059 ± 51% (100 μM ionomycin), (mean ± SEM n = 4-8). $InsP_6$, but not its non-physiological analogue, inositol hexasulphate, induced a marked accumulation of $^{45}Ca^{2+}$, much greater than that due to TRH or K^+, but still clearly less than that due to ionomycin. The effect of $InsP_6$ was concentration-dependent showing a statistically significant increment at 3 μM and above, and was saturable with a maximum response of 519 ± 70% increase over basal at 30 μM (n = 6). The $^{45}Ca^{2+}$ accumulation due to 20 μM $InsP_6$ was reduced by 78 ± 4% (n = 4) on inclusion of 100 μM digitonin in the EGTA wash medium. Cell viability (assessed with 0.2% Trypan Blue or 0.5 μg/ml fluorescein diacetate) was 93-100% following incubation with 150 μM $InsP_6$ for 30 min, (n = 4). The effect of $InsP_6$ could not be mimicked by Na_3PO_4, Na glutamate, EGTA, EDTA, 2,3-diphosphoglycerate or Na hydroxide (all at 1mM). At 150 μM, $InsP_6$ caused no change in the pH of the medium, although stock solutions of $InsP_6$ (K^+salt) were extremely alkaline.

The effect of 20 μM $InsP_6$ was unaltered by blockers of L-and N-type voltage-sensitive Ca^{2+} channels (nimodipine, 9 ± 11% inhibition at 1μM and ϖ-conotoxin, 15 ± 8% inhibition at 1 μM; n = 6 in each case). Polyvalent cations however showed the following IC_{50}'s (μM concentrations producing 50% inhibition): Gd^{3+}, 1.2 ± 0.5; La^{3+}, 5.7 ± 2.3; Cd^{2+}, 112 ± 16; Co^{2+}, 550 ± 75; Ni^{2+}, 956 ± 132. The low potency of Ni^{2+} is inconsistent with the involvement of a T-type Ca^{2+} channel. Replacement of Na^+ with N-methyl glucamine or the selective inhibitor of the Na^+/Ca^{2+} exchanger (5-(N-4-chlorobenzyl)-2',4'-dimethylbenzamil) at a concentration of 30 μM, had no effect on the response to $InsP_6$. It seemed unlikely that $InsP_6$ was acting by depolarising cells in view of the greater maximal response to $InsP_6$ than to K^+. Indeed when GH_3 cells were loaded with the potential-sensitive dye, 3,3'-dihexyl-oxacarbocyanine iodide, the change in fluorescence (excitation 484 nm, emission 510 nm) induced by 60 mM K^+ could not be mimicked by 20 μM $InsP_6$. Seeking support for the idea that cytosolic Ca^{2+} levels would be raised in response to $InsP_6$, further experiments were carried out with the Ca^{2+} fluorophore INDO-1 AM, with excitation at 332 nm and emission at 400 nm (Ca^{2+}-bound) and 483 nm (free form of INDO-1). Using GH_3 cells or rat hippocampal synaptosomes, 100 μM $InsP_6$ but not Ins $(SO_4)_6$, produced a clear rise in fluorescence at 400 nm or in the 400:483 nm ratio, comparable to the response to 60 mM K^+. Without cells present $InsP_6$ caused an increase in fluorescence of INDO-1 salt but this could be largely suppressed by 100μM Mn^{2+}. Using extracellular Mn^{2+} to suppress dye leakage artefacts, a clear response to $InsP_6$ still occurred with GH_3 cells or synaptosomes. Surprisingly, the response to 100 μM $InsP_6$ was similar even if preceded by 100 μM ionomycin. Unfortunately, $InsP_6$ (3-100μM) caused a clear concentration-dependent increase in emission at 400 nm when only medium (containing 1.5 mM Ca^{2+}), without cells or INDO-1 was present. Spectral analysis revealed a fairly uniform response from 350-600 nm,indicating a non-specific physical phenomenon.We then looked further at the possibility of low-solubility artefacts arising in the $^{45}Ca^{2+}$ experiments.

A hypotonic EGTA wash solution (2 mM EGTA, 6 mM HEPES) failed to release any of the $InsP_6$-induced $^{45}Ca^{2+}$ accumulation. Filters alone showed a small increase in $^{45}Ca^{2+}$ retention due to 100 μM $InsP_6$ but less than 10% of the response in the presence of GH_3 cells. The effect of $InsP_6$ on cellular Ca^{2+} accumulation was not reduced by carrying out the experiment at 0°C rather than 37°C and some 60-105% of the normal response was apparent in zero time blanks. Preincubation of cells at 80°C for 5 min failed to reduce the effect of $InsP_6$, although increasing the temperature to 100°C caused more than 50% reduction, as did pretreatment with 2% paraformaldehyde. Incubation under N_2, with 100 μM carbonyl cyanide m-chlorophenyl hydrazone reduced the effect of 100 μM $InsP_6$ by around 60%. Although obvious turbidity could rarely be detected by eye, 10-300 μM $InsP_6$ in the experimental medium showed clear concentration-dependent increases in OD_{520}. Another anion which forms low-solubility complexes with Ca^{2+}, oxalate, had similar effects from 100-10000 μM. When $^{45}Ca^{2+}$ experiments were carried out with oxalate, large accumulations of $^{45}Ca^{2+}$ were induced at equivalent concentrations. Clear accumulation of $^{45}Ca^{2+}$ in the presence of oxalate was seen with filters alone. In the presence of GH_3 cells (just like with $InsP_6$) there was a marked increase (in the order of 5 fold) in the amount of $^{45}Ca^{2+}$ retained. All of the effects of $InsP_6$ observed in our laboratory can thus be explained as physical phenomena without the need to invoke bio-activity.

The combination of chelation and solubility properties shown by $InsP_6$ and $InsP_5$ but to a much lesser extent by lower inositol phosphates [4] is bound to confound any investigation of their influence on cellular Ca^{2+} movements. In our opinion, both the present results and those provided in recent reports on $^{45}Ca^{2+}$ accumulation [5,6] and Ca^{2+} fluorimetry [7] cannot be considered with any validity to demonstrate biological effects of inositol polyphosphates on cellular Ca^{2+} movements.

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Differences in the effect of putative activators of protein kinase C on secretion of pituitary hormones

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Secretion of pituitary hormones *in vitro* can be induced by activation of protein kinase C (PKC) (Johnson and Mitchell, 1989). Using several putative activators and inhibitors of PKC, we investigated the possibility that PKC-mediated regulation of hormone secretion may be differentially organised in distinct pituitary cell types. Anterior pituitary glands were removed from long term (4 weeks) ovariectomised cob wistar rats and hemisected. Release of luteinizing hormone (LH) and growth hormone (GH), *in vitro*, was measured as previously described (Johnson & Mitchell,1989). Pituitary LH release progressively increased over 3 consecutive hourly incubations with 300nM phorbol 12,13-dibutyrate (PDBu), reaching a level 192±21% (n=16) of basal secretion in the third hour. In contrast, GH was rapidly released by PDBu with a maximal response in the first hour of 558±95% (n=16) of basal secretion. PDBu-induced release of both LH and GH was unaffected by the PKC inhibitor H7(30μM) but was attenuated in the presence of staurosporine (300nM). Selective inhibition by staurosporine but not H7 of certain PKC actions has been described previously in the regulation of L-type calcium channels by PKC (Johnson *et al.*,1989). In the present experiments, 200μM 1,2-dioctanoyl *sn*-glycerol (DOG) induced a small release of LH but not GH. Luteinizing hormone release induced by DOG was inhibited by staurosporine but was unaffected by H7. Release of LH could also be evoked by 300μM arachidonic acid (AA), but this effect was unaffected by PKC inhibitors. Release of GH was reduced by AA, an effect which is also unaltered by PKC inhibitors.

The release of LH and GH from pituitary tissue obtained from ovariectomized rats can be influenced by activation of PKC. However, the lack of effect of H7 on LH release shown here contrasts with those effects observed on PDBu induced LH release from tissue obtained from intact pro-oestrous rats (Johnson & Mitchell,1989.). It appears that AA can exert stimulatory (LH) or inhibitory (GH) effects on hormone secretion by means other than activation of PKC. This is consistent with evidence suggesting a role for AA metabolites in the control of LH and GH release (Naor *et al*, 1983; Schweitzer *et al*, 1990).

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DIFFERENTIAL INVOLVEMENT OF PHOSPHOLIPASE A_2 IN PHORBOL ESTER-INDUCED LUTEINIZING HORMONE AND GROWTH HORMONE RELEASE FROM RAT ANTERIOR PITUITARY TISSUE.

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SUMMARY

The protein kinase C (PKC) activator, phorbol 12,13-dibutyrate (PDBu) can induce the release of both luteinizing hormone (LH) and growth hormone (GH) from proestrous rat anterior pituitary pieces, *in vitro*. Phorbol 12,13-dibutyrate-induced LH, but not GH release was readily inhibited by the phospholipase A_2 (PLA_2) inhibitors, quinacrine, *p*-bromophenacyl bromide and aristolochic acid. Furthermore, PDBu could induce a quinacrine-sensitive release of [^{3}H]arachidonic acid ([^{3}H]AA) from pre-labelled anterior pituitary tissue. The protein synthesis inhibitor, cycloheximide, inhibited the release of both [^{3}H]AA and LH that was induced by PDBu, whereas, LH release induced by the PLA_2 activator, melittin, was cycloheximide-insensitive. These results suggest that PKC activators may induce LH but not GH release from anterior pituitary tissue by a mechanism involving activation of PLA_2 by a process which is reliant on protein synthesis.

Key words: protein kinase C, phospholipase A_2, luteinizing hormone, growth hormone

INTRODUCTION

Activation of the Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C (PKC) is important for cellular responses to certain hormones (Nishizuka, 1984, 1988) including luteinizing hormone-releasing hormone (LHRH) (Johnson et al., 1992a). Protein kinase C exists as a family of at least 8 different isoforms, each having distinct tissue distributions suggesting that each isoform may have discrete cellular targets and actions (Nishizuka, 1988; Osada et al., 1990). Consistent with this hypothesis, PKC forms which display distinct pharmacology, with respect to PKC activators (such as phorbol esters) and PKC inhibitors, appear to be involved in the induction of LHRH self-priming, luteinizing hormone (LH) release and growth hormone (GH) release in proestrous rat anterior pituitary tissue (Johnson et al., 1989, 1992a, 1992b; Thomson et al, 1992). Since the temporal patterns of phorbol ester-induced LH and GH release are different (Johnson et al, 1989) the sequence of events that lead from PKC activation to hormone release from gonadotropes is probably very different from that in somatotropes. Thus, the PKC forms that induce LH and GH release may phosphorylate different cellular targets. One potential target for the PKC(s) which induce LH release may be phospholipase A_2 (PLA_2), or an intermediate leading to the activation of PLA_2.

The release of LH can be induced from primary cultures of rat gonadotropes by the PLA_2 activator, melittin, and by exogenously added snake venom PLA_2 (Kiesel et al., 1985). The actions of LHRH are accompanied by an increased release of arachidonic acid (AA), a product of PLA_2 action, and AA metabolites (Naor and Catt, 1981; Catt et al., 1985). Furthermore, exogenously added AA, and several AA metabolites, can induce LH release from dispersed anterior pituitary cells (Naor et al., 1983;

Hulting et al., 1984; Kiesel et al., 1987). In other cell types, phorbol esters can induce an increase in free AA levels (Halenda and Rehm, 1987; Parker et al., 1987) suggesting that PLA_2 activity may be modulated as a consequence of PKC activation. In these experiments, we examined a possible role for PLA_2 in the mechanism of PKC-induced LH and GH release from proestrous rat anterior pituitary and the possible involvement of protein synthesis in this response.

MATERIALS AND METHODS

Animals

Adult female COB Wistar rats (200 g - 250 g body weight; Charles River UK Ltd) were maintained under controlled lighting and temperature with free access to food pellets (CRM, Labsure, Manea, Cambs, UK) and tap water. Vaginal smears were examined and rats with a minimum of two regular estrous cycles were anaesthetised with sodium pentabarbitone (30 mg/kg; Sagatal; May and Baker Ltd Dagenham, Essex) by 11.30 am on proestrus.

Chemicals

Luteinizing hormone-releasing hormone, cycloheximide, quinacrine dihydrochloride, (Sigma Chemical Co, Poole, Dorset, UK) 1-(5-isoquinolinesulphonyl)-2-methyl-piperazine dihydrochloride (H7) (Gibco Brl, Paisley, Scotland) and aristolochic acid sodium salt (Biomol, c/o Semat, St Albans, Herts, UK) were made up as stock solutions in distilled H_2O. Staurosporine (Novabiochem, Nottingham, UK) and Ro 31-8220 (a gift from Roche Products Ltd, Welwyn Garden City, Herts UK) were made up as stock solutions in dimethylformamide (DMF). ρ-Bromophenacyl bromide (BrPheBr) (Sigma) was made up as a stock solution in ethanol. The maximum concentration of DMF or ethanol (0.05 % v/v) was used in

control experiments and had no effect on either LH or GH release or [^{3}H]AA release. [5,6,8,9,11,12,14,15-^{3}H(N)]Arachidonic acid ([^{3}H]AA) (specific activity approximately 240 Ci/mmol) was purchased from Du Pont, Dreieich, Germany. All other chemicals were of analar grade and were purchased from BDH (Poole, Dorset, UK).

Hormone Release Experiments

The methods used were based upon those described by Pickering and Fink (1976). Anterior pituitary glands were hemisected and each hemipituitary placed into a flask containing 2 ml of pre-warmed (37°C) and gassed (95%O_2/5%CO_2) Hepes-buffered minimal essential medium (MEM) (Gibco Brl, Paisley, UK) with Earles' salts.

After a 30 minute pre-incubation period in a shaking water bath (37°C, 95%O_2/5%CO_2), and every subsequent hour thereafter, the medium was replaced. In the initial, basal hour, the medium contained either an inhibitor or no drug. For each subsequent hour, medium also contained PDBu (300 nM).

The medium removed at the end of each incubation period was kept at -20°C until it was radioimmunoassayed for LH and GH (Niswender *et al*, 1968; Daane and Parlow, 1971; Johnson *et al*, 1988). The standards used were NIADDK-rat LH-RP2 and NIADDK-rat-GH-RP2.

[^{3}H]Arachidonic Acid Release Measurements

[^{3}H]Arachidonic acid release was measured from pre-labelled anterior pituitary tissue as follows. Anterior pituitary glands were removed, hemisected and each hemipituitary cut into two equal quarters. Pairs of pituitary quarters were then placed into a silanised flask containing 1 ml of pre-warmed (37°C) and pre-gassed (95%O2/5%CO2) MEM and pre-incubated in a shaking water bath (37°C, under

95%O_2/5%CO_2) for 30 minutes. The medium was then replaced with fresh MEM containing 0.5 μCi of [^{3}H]AA and the tissue incubated for a further 2 hours. The tissue was then washed three times in MEM containing 1% essential fatty acid-free bovine serum albumin (BSA; Sigma) to remove unesterified [^{3}H]AA.

For studies of [^{3}H]AA release, the tissue was incubated in MEM + 0.5% BSA (as a trap for released [^{3}H]AA) in the presence or absence of PDBu. After 15 min, the incubation medium was removed and the medium [3H]AA levels were determined by lipid extraction and reverse-phase liquid chromatography on octadecyl silica (ODS) using the solvent system described by Powell (1982) as follows. Ethanol was added to the incubation medium to give a final ethanol concentration of 30% v/v. The medium was centrifuged (10', 5°C, 3000 g) and the supernatant was acidified with 1M HCl to pH 3. The acidified medium (4 ml) was loaded onto a pre-washed (5 ml ethanol followed by 5 ml of dH_2O) 1.25 cm ODS column (sep-pak C_{18} cartridge, Waters Chromatography, Watford, Hertfordshire, UK). Solvents were then passed through the column in the following order; 30% ethanol (20 ml), distilled H_2O (20 ml), petroleum ether (10 ml), petroleum ether: $CHCl_3$ (1:1, 20 ml). The solvent fraction taken to represent [^{3}H]AA released from the tissue (petroleum ether: $CHCl_3$) contained over 83% of standard authentic [^{3}H]AA that had been incubated with inactivated tissue (previously homogenised in 30% ethanol) and carried through the extraction procedure.

The radioactivity remaining in the tissue was determined by washing the tissue twice in 1 ml MEM containing 1% BSA. The tissue was then homogenised in 1 ml MEM + 0.5% BSA using a hand held tissue grinder and diluted in ethanol to a final concentration of 30% v/v. The homogenate was then spun (10 min, 5°C, 3000 g) and the supernatant

acidified to pH 3. Aliquots of the supernatants from the tissue homogenate and the incubation media were counted and the total amount of label incorporated into the tissue was determined.

In the experiments where the effects of inhibitors were examined on PDBu-induced [^{3}H]AA release, the tissue was pre-incubated for 15 minutes with the appropriate inhibitor, the medium was discarded and the tissue incubated with medium containing, in addition, PDBu for a further 15 min as described above.

Statistics

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical analyses were carried out using the Mann Whitney U-test. The EC_{50} values (the effective concentration which produces 50 % of the maximal response) for the PDBu and the IC_{50} values (the concentration which inhibits 50 % of the maximal response) for the kinase inhibitors and PLA_2 inhibitors were obtained from fitting the data with the non-linear, error-weighted iterative curve fitting programme, P.fit (Biosoft, Cambridge, UK) and are given as mean ± S.E.M.

RESULTS

Over consecutive hourly incubations with PDBu (300 nM), both LH and GH release from proestrous rat hemipituitaries were significantly increased above basal h levels, but with different temporal patterns (Figure 1). That is, the release of GH was maximal during the 1st h of PDBu incubation (Figure 1b), whereas, LH release did not increase significantly above basal h levels until the 2nd h of PDBu incubation and then increased further in the 3rd h (Figure 1a). Third h PDBu-induced LH release was significantly inhibited in the presence of the PLA_2 inhibitor quinacrine (Markus and Ball, 1969; Löffler et al., 1985) (Figure 1a and 2) with an IC_{50} value of 20 ± 9 μM. In contrast, GH release measured during any hour of phorbol incubation was unaltered by quinacrine (Figure 1b), even at concentrations as high as 100 μM where PDBu-induced LH release was decreased to levels which were approximately 10% of control (Figure 2). Aristolochic acid (100 μM) and BrPheBr (50 μM), at concentrations which are reported to cause over 50 % inhibition of PLA_2 activity in whole cells but which have minimal effects on other enzymes (Drenth et al., 1976; Vishwanath et al., 1988), also inhibited PDBu-induced LH release but not PDBu-induced GH release (Table 1). The actions of these inhibitors suggest that PLA_2 may be a target for the PKC(s) which induce LH release, but not those which induce GH release.

Neither quinacrine (100 μM), aristolochic acid (150 μM), nor BrPheBr (50 μM) had any significant effect on baseline LH or GH release when measured over several hours of incubation.

The influence of PKC activators on anterior pituitary PLA_2 activity was examined, more directly, by measuring [^{3}H]AA release from pre-labelled anterior pituitary tissue. After a 15 min incubation with PDBu

(100 nM - 1 μM), [^{3}H]AA release from proestrous tissue was significantly increased above baseline levels (Figure 3) (EC_{50} = 79 ± 27 nM). An increase in the release of free [^{3}H]AA levels from cellular phospholipids may result from the action of PLA_2 or by the sequential actions of phospholipase C, which releases diacylglycerol (DAG), and DAG lipase, which will catalyse the removal of a fatty acid from the glycerol backbone of DAG (Naor, 1991). To determine the pathway leading to [^{3}H]AA release in this system, we examined the effect of quinacrine and the DAG lipase inhibitor, RHC 80267 on the PDBu response. The inhibitors were used at concentrations reported to selectively inhibit the activity of their targets with minimum side-effects (Sutherland and Amin, 1982; Hoffman et al., 1982). Quinacrine (50 μM), but not RHC 80267 (80 μM), inhibited PDBu (300 nM)-induced [^{3}H]AA release (Table 2), indicating that this phorbol ester effect on proestrous rat anterior pituitary tissue [^{3}H]AA release occurs through a PLA_2-dependent route.

The PKC inhibitors, staurosporine and Ro 31-8220 (Davis et al, 1989) both fully inhibited 300 nM PDBu-induced [^{3}H]AA release from pre-labelled tissue with IC_{50} values of 28 ± 18 nM and 7 ± 3 μM respectively (Figure 4a and 4b). Phorbol ester-induced AA release was also inhibited by H7 (Figure 4c), but in a clearly biphasic manner. Although more than 50% of the response to PDBu was blocked by H7 concentrations as little as 1 μM, no further inhibition was observed until the H7 concentration was then further increased by over 30-fold, suggesting that the PKC form mediating some 40% of this response was highly resistant to H7. These results indicate that both H7-resistant and -sensitive forms of PKC may be involved in the mechanism of PDBu-induced [^{3}H]AA release. Neither quinacrine (50 μM), RHC 80267 (200 μM), staurosporine (300 nM), H7 (100

μM) nor Ro 31-8220 (30 μM) had any significant effect on baseline [^{3}H]AA release (data not shown).

Since a previous report has indicated that phorbol ester-induced LH release from rat anterior pituitary pieces is dependent upon protein synthesis (Bourne et al., 1989), we investigated the possible involvement of protein synthesis in the mechanism of PKC-activator induced PLA_2 activation. The protein synthesis inhibitor, cycloheximide (Obrig et al., 1971) (50 μM) significantly inhibited PDBu (300 nM)-induced LH release, but not GH release during all hours of incubation (Figure 5). Cycloheximide also inhibited PDBu-induced [^{3}H]AA release (Table 3). In contrast, cycloheximide had no effect on LH release induced by the PLA_2 activator, melittin (Habermann, 1972) during any hour of incubation (Figure 6). This suggests that the phase of protein synthesis essential for phorbol ester-induced LH release may occur following the activation of PKC, but prior to the actions of PLA_2.

DISCUSSION

Previous reports suggest that stimulation of rat gonadotrope LHRH receptors can lead to activation of PKC (Johnson et al., 1992a) and of PLA_2 (Naor and Catt, 1981) leading to LH release. The present evidence suggests, that in proestrous rat hemipituitary pieces, activation of PLA_2 may be required for the mechanism of PKC activator-induced LH but not GH release. The PLA_2 inhibitors, quinacrine, BrPheBr and aristolochic acid blocked PDBu-induced LH release, but not GH release (Figures 1, 2 and Table 1), with potencies similar to their effects on PLA_2-mediated responses in other cell types (Löffler et al., 1985; Rosenthal et al., 1989; Vallee et al., 1978). Notably, the concentration of quinacrine which was reported to inhibit long-term secretory responses to LHRH in primary

cultures of anterior pituitary cells (IC_{50} = 20 μM) (Naor and Catt, 1981) is within the range described here for the inhibition of PDBu-induced LH release from proestrous rat anterior pituitary pieces (IC_{50} = 20 ± 9 μM).

The specificity of both quinacrine and BrPheBr as inhibitors of PLA_2 has been questioned in a number of studies (Irvine, 1982; Dise et al., 1982; Chang et al., 1987). However, quinacrine does not inhibit LH release induced by depolarising concentrations of K^+ or by Ca^{2+} ionophores (unpublished observations), suggesting that a non-specific action of this drug is unlikely to be responsible for its effects on PDBu responses. Although BrPheBr and quinacrine are putative inhibitors of PLC activity (Hofmann et al., 1982), these compounds are unable to prevent the presumed PLC-dependent, initial LH release response to LHRH, measured in proestrous rat tissue (unpublished observations). In addition, at the concentrations used here, these compounds have been reported to inhibit PLA_2 in other systems without affecting PLC activity (Lazarewicz *et al*, 1988). Aristolochic acid can inhibit the activity of PLA_2 from snake venom, human synovial fluid and platelets (Vishwanath et al., 1988; Rosenthal et al., 1989) by interacting directly, but non-covalently with the enzyme, inducing a change in the secondary structure of the protein (Vishwanath et al., 1987). This mechanism of interaction between PLA_2 and aristolochic acid would imply that this agent may have specificity for PLA_2 over other phospholipases.

In agreement with this pharmacological evidence, suggesting that PKC activation by phorbol esters modulates anterior pituitary PLA_2 activity, PDBu induced a relatively rapid (15 min) increase in [^{3}H]AA release from proestrous rat anterior pituitary pieces (Figure 3); a response which was blocked by inhibitors of PLA_2, but not DAG lipase action (Table 2). Unfortunately, due to the small population of gonadotropes in anterior

pituitary tissue, it was not possible to measure changes in the cellular content of [^{3}H]AA after stimulation. The exact relationship between medium [^{3}H]AA levels (as measured in these experiments) and intracellular-free AA levels within the gonadotrope is therefore unclear.

Although, PKC-dependent phorbol ester-modulation of PLA_2 activity has been described in other cell types (Parker et al., 1987; Akiba et al., 1990), there are some suggestions that phorbol esters may directly activate phospholipases (Billah et al., 1989; Billah and Anthes, 1990). It is unlikely that direct activation of PLA_2 can account for the effects of PDBu on anterior pituitary [^{3}H]AA release since this effect required protein synthesis, implying that the mechanism of activation is complex. Furthermore, PDBu-induced [^{3}H]AA release from hemipituitaries was readily inhibited by staurosporine and Ro 31-8220, suggesting that PKC is involved in this response (Figure 4). The PDBu-induced [^{3}H]AA release response showed two components of inhibition by H7, suggesting that both H7-resistant and H7-sensitive kinases may participate in this response. We, and others, have previously reported that certain cellular responses to phorbol esters display unusual resistance to H7 (Watson et al., 1988; Johnson and Mitchell, 1989; Nakadate et al., 1989; MacEwan and Mitchell, 1991; Johnson et al., 1992a, 1992b; Thomson et al., 1992). Furthermore, in enzyme assays of anterior pituitary cytosol PKC activity, we have detected a PDBu activated, phosphatidylserine-dependent, Ca^{2+}-independent kinase activity which is relatively resistant to inhibition by H7 (MacEwan et al., 1992). Interestingly, we have found that activation of an H7-resistant, but staurosporine- and Ro 31-8220-sensitive, PKC is required for the induction of LHRH priming (Johnson et al., 1992a) and that this kinase may bring about the priming response by a mechanism involving increased PLA_2 activity in the gonadotrope (unpublished observations).

Phorbol ester-induced LH release was very markedly inhibited by cycloheximide (Figure 5a), consistent with a previous observation (Bourne et al., 1989), and suggested that synthesis of an unknown critical protein(s) is elicited in these cells by PKC activation. The present results provide evidence which suggests that the protein synthesis-dependent step is the mechanism by which PKC modulates PLA_2 activity. Phorbol 12,13-dibutyrate-induced [^{3}H]AA release was blocked by cycloheximide, whereas, melittin-induced LH release was not affected by the inhibitor. However, melittin can induce voltage-dependent ion conductances in planar lipid bilayers and, at high concentrations, can cause cell membrane damage (Tosteson and Tosteson, 1981), thus melittin-induced LH release may not occur by an entirely PLA_2-dependent process. Nevertheless, these results do suggest that PKC modulates PLA_2 activity in a protein synthesis-dependent manner. In smooth muscle and epithelial cells, PKC can regulate the rapid synthesis of an endogenous, melittin-like protein which activates PLA_2 (Clark et al., 1987, 1991). This PLA_2-activating protein (PLAP) has been shown to have a role in the leukotriene D_4 receptor signalling system (Crooke et al., 1989). Further investigations are required to examine the possible involvement of PLAP in the control of gonadotrope PLA_2 activity by PKC.

Arachidonic acid, and its lipoxygenase and epoxygenase metabolites, have been suggested to have a role in the mechanism of LH release (Naor and Catt, 1981; Naor et al., 1983; Hulting et al., 1984; Kiesel et al., 1987, Snyder et al, 1983). Arachidonic acid has a number of cellular actions, and any combination of these may be involved in the mechanism of PKC activator-induced LH release. For example, AA can induce Ca^{2+} release from non-mitochondrial stores (Wolf et al., 1986; Chan and Turk, 1987) resulting in a promotion of Ca^{2+}-dependent processes such as

gonadotropin release. Arachidonic acid has been reported to act both directly and synergistically with other lipid products in the activation of certain PKC isoforms (McPhail et al., 1984; Shearman et al., 1991). In addition, AA, and perhaps lipoxygenase metabolites, can modulate the activity of voltage-sensitive ion channels permeable to Ca^{2+} or K^{+} (Kim and Clapham, 1989; Kurachi et al., 1989; Ordway et al., 1991). The relevance of these actions of AA in the mechanism of PDBu-induced LH release is unclear and obviously requires further investigation. Interestingly, endogenous AA production can be dissociated from exocytosis in certain cell types (Churcher et al., 1990; Morgan and Burgoyne, 1990; Cockcroft, 1991) suggesting that AA may not be a direct mediator of secretion. Indeed, in cell-free systems, AA can enhance fusion of secretory granules into membranes in a Ca^{2+}-dependent manner modulating exocytosis (Creutz, 1981). It is possible that an equivalent action of AA in gonadotropes may enhance Ca^{2+}-induced LH release.

Neither cycloheximide nor any PLA_2 inhibitor tested blocked PDBu-induced GH release indicating that neither protein synthesis nor PLA_2 activation is required for the mechanism of PKC activation-induced GH release. Instead PDBu-induced GH release is suggested to occur by a route involving direct modulation of 'L'-type voltage-sensitive Ca^{2+} channels by PKC (Johnson et al., 1991).

In conclusion, these experiments show that PDBu-induced LH but not GH release from proestrous rat anterior pituitary pieces, *in vitro*, requires PLA_2 activation and protein synthesis. The relationship of the PKC forms which control PLA_2 activity to the known isoforms of PKC is unclear. However, it would appear that more than one form of PKC (both H7-resistant and -sensitive types) is involved in the process of PKC activator -induced [^{3}H]AA release (as shown here) and in PKC-activator-

induced LH release (Johnson et al. ,1989, 1992b). Thus, the PKCs that induce LH and GH release, as well as being pharmacologically distinct (Johnson et al., 1989, 1992b), may have distinct cellular targets which may, in part, account for the difference in the time course of PDBu-induced release for each of these hormones.

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TABLE 1

The effect of PLA_2 inhibitors on PDBu-induced LH and GH release from proestrous rat anterior pituitary tissue

Hemipituitaries were incubated for a basal h either in with no drug or with a PLA_2 inhibitor (BrPheBr, 50 μM) or aristolochic acid (100 μM). In the following hours (1st h, 2nd h, 3rd h), the incubation medium contained, in addition, PDBu (300 nM) or no drug (baseline). The data shown here represents LH release measured during the 3rd h of incubation and GH release measured during the 1st h of incubation with PDBu. The statistical significance of the effects of PLA_2 inhibitors on PDBu-induced hormone release is represented by * ($p \leq 0.05$, Mann-Whitney U-test). Values shown are the means ± S.E.M. and the number of determinations are shown in parentheses.

	LH release (μg/l)	GH release (μg/l)
baseline	4.9 ± 0.9 (4)	259.1 ± 14.1 (4)
PDBu	32.3 ± 2.7 (6)	1964.3 ± 108.8 (6)
PDBu + BrPheBr	*15.8 ± 2.1 (4)	2072.3 ±368.7 (4)
PDBu + aristolochic acid	*14.2 ±1.5 (4)	2239 ± 207.8 (4)

TABLE 2

The effect of quinacrine and RHC 80267 on PDBu-induced [^{3}H]AA release from pre-labelled proestrous rat anterior pituitary pieces

[^{3}H]Arachidonic acid-labelled pairs of anterior pituitary quarters were pre-incubated for 15 minutes in medium only, or with quinacrine (50 μM) or RHC 80267 (80 μM), the medium was discarded and replaced with fresh medium containing either no drug (baseline) or PDBu (300 nM), or PDBu and either quinacrine or RHC 80267. After the second 15 min incubation, the medium was removed and the [^{3}H]AA was extracted. The statistical significance of the effects of quinacrine and RHC 80267 on LHRH-induced [^{3}H]-AA release was determined (*$p < 0.05$, Mann-Whitney U-test). Values shown are the means ± S.E.M. and the number of determinations are shown in parentheses.

	[^{3}H]AA release (% total label incorporated)
baseline	1.59 ± 0.07 (10)
PDBu	2.73 ± 0.16 (8)
PDBu + quinacrine	*1.65 ± 0.02 (5)
PDBu + RHC 80267	2.53 ± 0.22 (4)

TABLE 3

The effect of cycloheximide on PDBu-induced [^{3}H]AA release from proestrous rat anterior pituitary pieces

Pairs of pituitary quarters, pre-labelled with [^{3}H]AA, were pre-incubated for 15 min in medium containing either no drug or cycloheximide (50 μM). The medium was discarded and replacement medium contained, in addition, either no drug (baseline) or PDBu (300 nM). After incubation for 15 min, the medium was removed and [^{3}H]AA release was determined. Phorbol 12,13-dibutyrate-induced [^{3}H]AA release was significantly inhibited by cycloheximide (*$p < 0.05$, Mann-Whitney U-test). Values are means ± S.E.M. for the number of determinations shown in the parentheses.

	[^{3}H]AA release (% label incorporated)
baseline	1.04 ± 0.07 (10)
PDBu	1.65 ± 0.10 (8)
PDBu + cycloheximide	*1.16 ± 0.09 (8)

FIGURE 1

Effect of quinacrine on the time-course of PDBu-induced LH and GH release from proestrous rat hemipituitaries

Hemipituitaries were incubated for an initial basal h either with no drug (open bars) or with quinacrine (50 μM) (hatched bars). In the following consecutive hourly incubations (1st h, 2nd h, 3rd h), the incubation medium contained, in addition, PDBu (300 nM). The statistical significance of the inhibitory effects of quinacrine on PDBu-induced hormone release at each incubation period was determined (*$p < 0.05$, Mann-Whitney U-test). Data are the means ± S.E.M. for 4 - 6 determinations.

FIGURE 2

Concentration response curve for the effect of quinacrine on PDBu-induced LH and GH release from proestrous rat hemipituitaries

Hemipituitaries were incubated for a basal h in either medium only or with various concentrations of quinacrine (1 - 100 μM). Over the following consecutive hourly incubations, the medium contained, in addition, PDBu (300 nM). The data represents LH release (●) measured during the 3rd h of PDBu incubation and GH release (■) measured in the 1st h of phorbol incubation. Quinacrine caused a dose-dependent inhibition of PDBu-induced LH but not GH release (*$p < 0.05$, Mann-Whitney U-test). Each point on the graph represents the mean ± S.E.M. for 4 - 8 determinations.

FIGURE 3

Concentration response curve for the effect of PDBu on [^{3}H]AA release from pre-labelled proestrous rat anterior pituitary tissue.

Anterior pituitary tissue, which had been pre-labelled with [^{3}H]AA and then extensively washed, was incubated in medium containing various concentrations of PDBu (10 nM- 1 μM). The levels of [^{3}H]AA released into the medium were then determined. The statistical significance of the effect of PDBu was determined (*$p < 0.05$, Mann-Whitney U-test). Data are means ± S.E.M. for 6 - 8 determinations.

FIGURE 4

Concentration-response curves for the effect of (a) staurosporine, (b) Ro 31-8220 and (c) H7 on PDBu-induced [^{3}H]AA release from proestrous rat anterior pituitary tissue

Pairs of pre-labelled anterior pituitary quarters were pre-incubated for 15 minutes with medium only or the appropriate concentration of PKC inhibitor. The medium was discarded and replaced with fresh medium containing either no drug (basal) or PDBu (300 nM) only or PDBu with various concentrations of inhibitor. After 15 minutes, [^{3}H]AA release into the medium was determined. Both staurosporine and Ro 31-8220 fully reversed the effect of PDBu. However, PDBu-induced [^{3}H]AA release apparently displayed 2 components of inhibition by H7. Each point on the graphs represents the mean ± S.E.M. for 4 - 8 determinations.

FIGURE 5

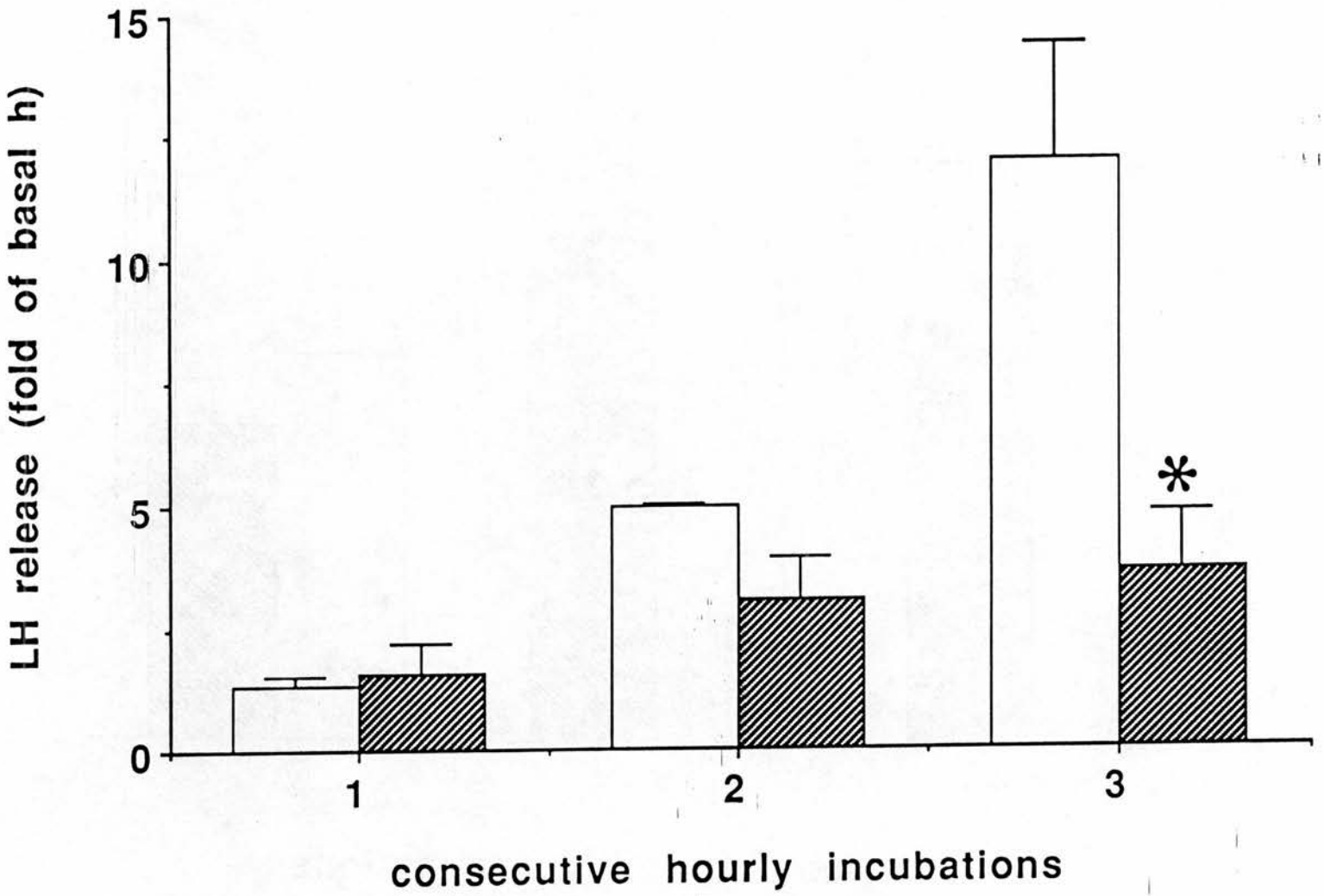
Effect of cycloheximide on PDBu-induced LH and GH release from proestrous rat anterior pituitary tissue

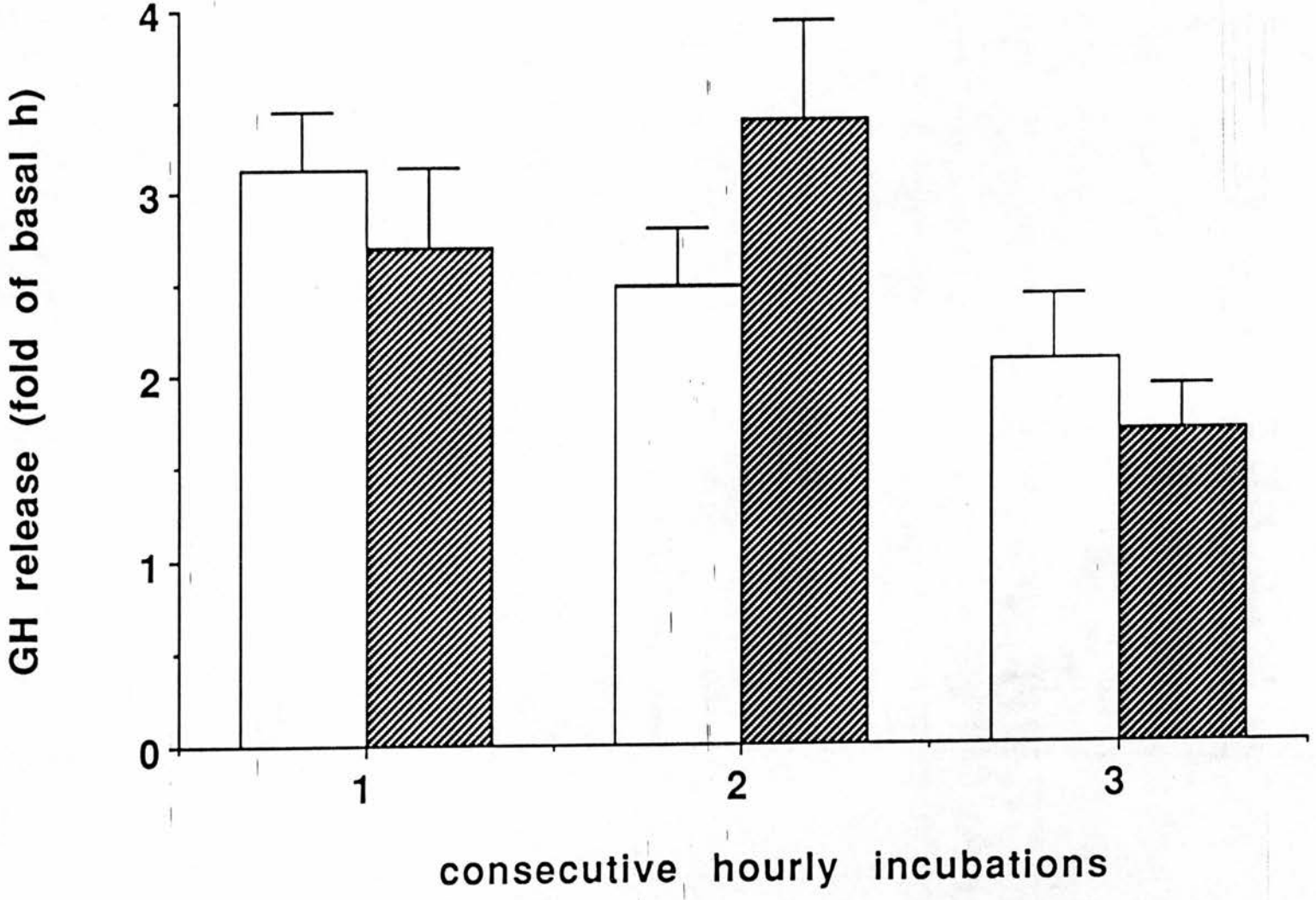
Hemipituitaries were incubated for a basal h in either medium only (open bars) or with cycloheximide (50 μM, hatched bars). Over a further 3 consecutive hourly incubations, the medium contained, in addition, PDBu (300 nM). Cycloheximide inhibited 2nd and 3rd h PDBu-induced LH but not GH release (*$p < 0.05$). The S.E.M. values for the cycloheximide treated samples are within a range of 0.4 to 0.6 μg/l and are not obvious from the scale used here. Each point on the graph represents the mean ± S.E.M. for 6 determinations.

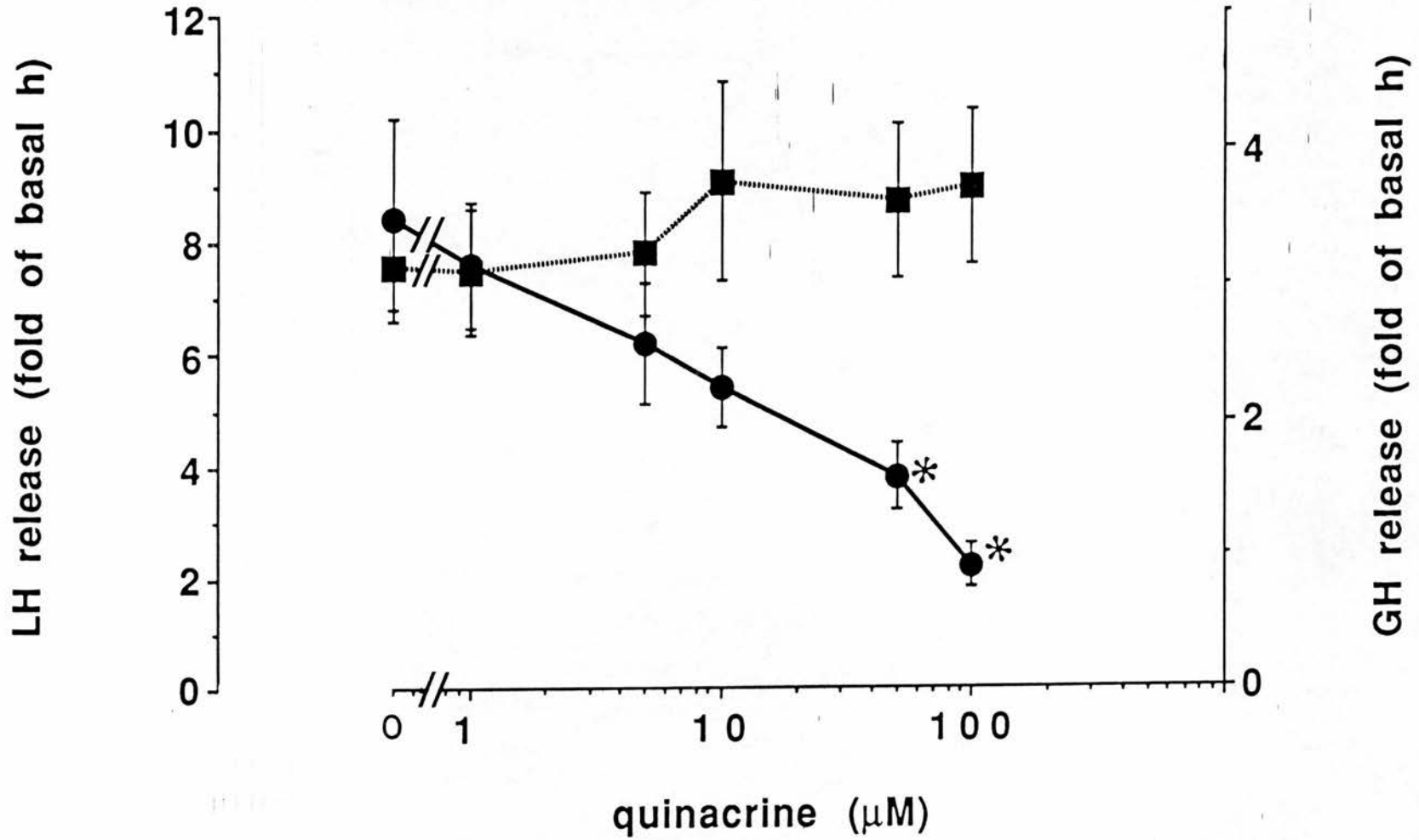
FIGURE 6

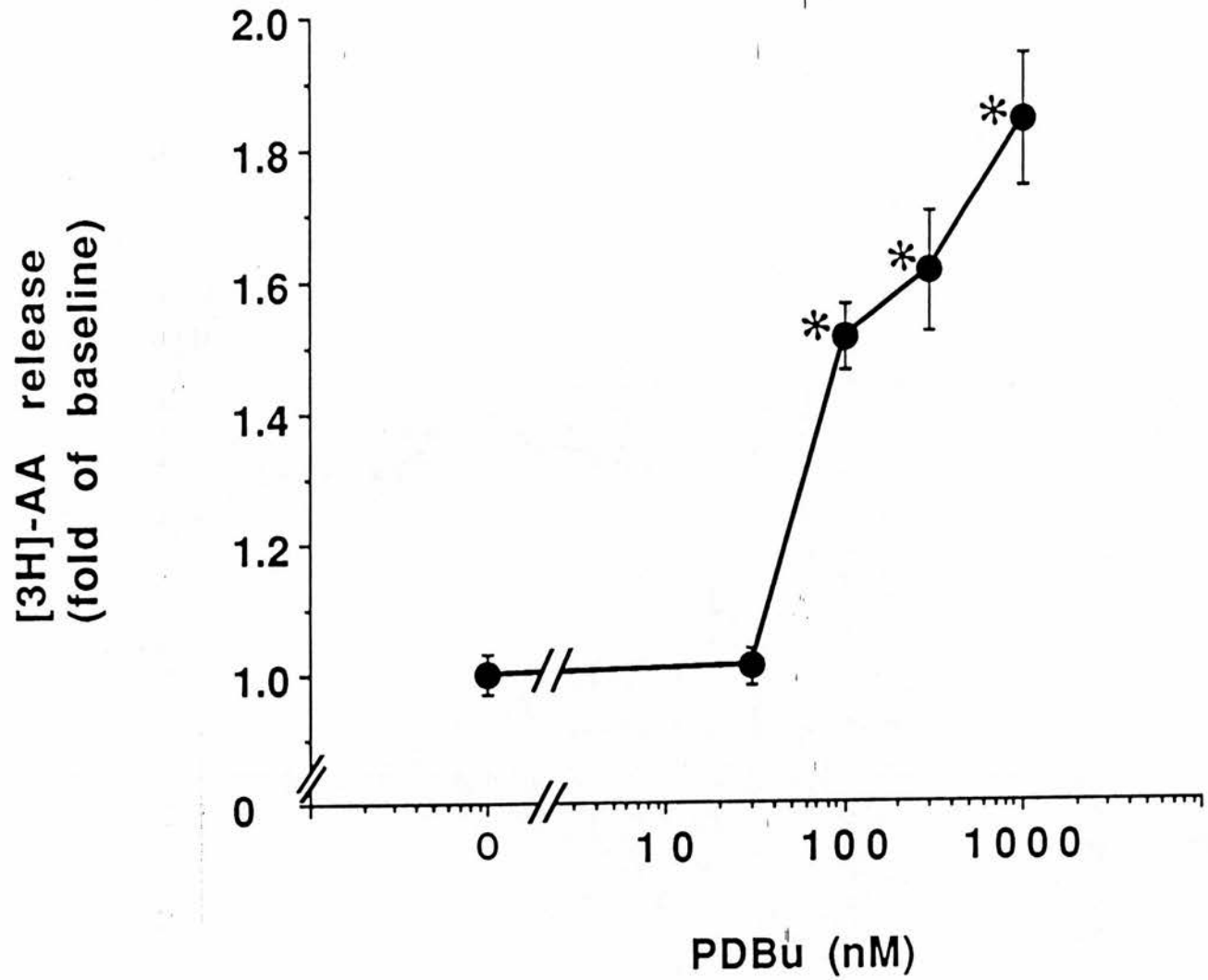
Effect of cycloheximide on melittin-induced LH release from proestrous rat hemipituitaries

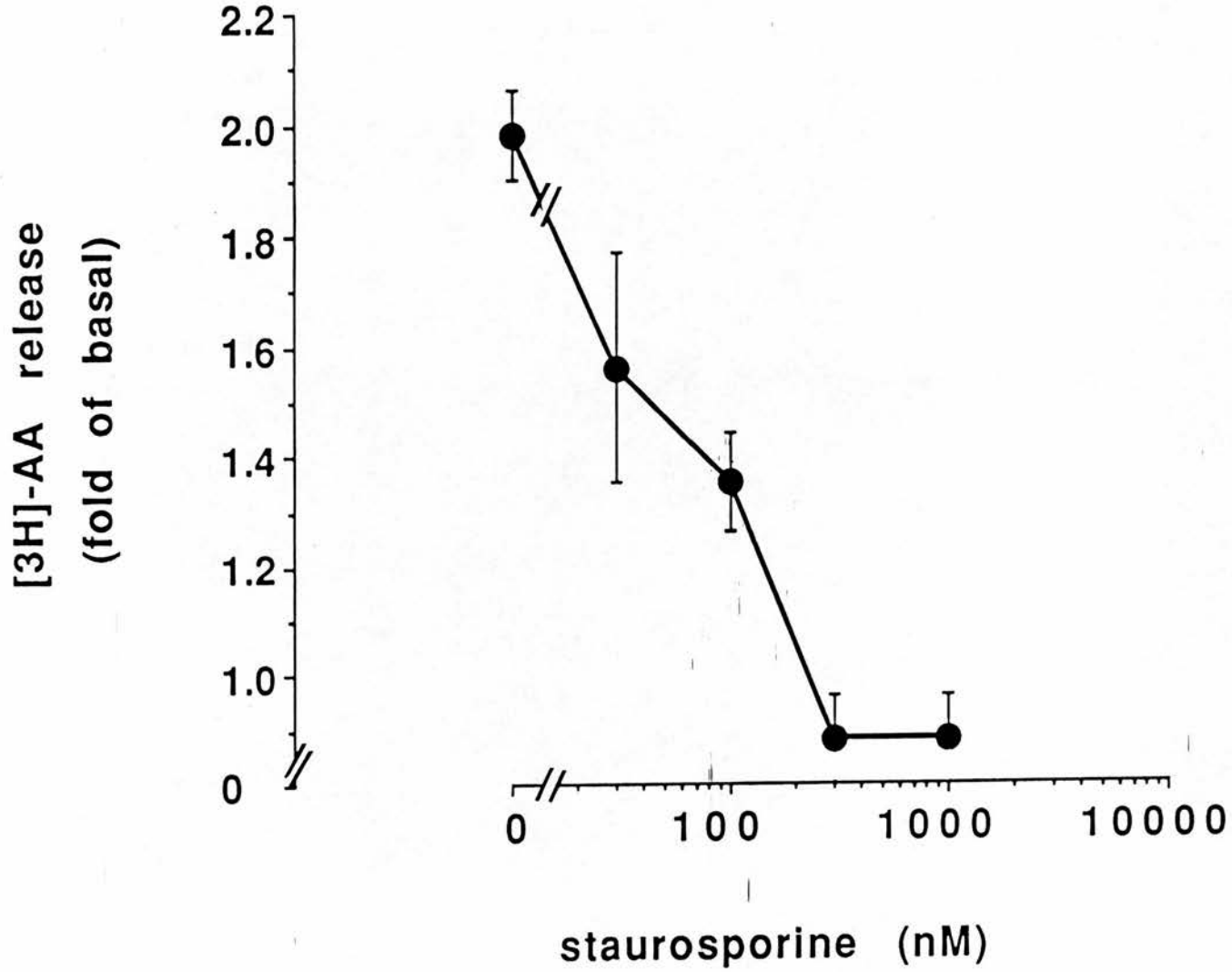
Hemipituitaries were incubated for a basal h in medium containing either with no drug (open bars) or cycloheximide (50 μM, hatched bars). In the following consecutive hourly incubations, the medium contained, in addition, melittin (3 μM). Melittin-induced LH release was clearly unaffected by the presence of cycloheximide. Data are the means ± S.E.M. for 4 determinations.

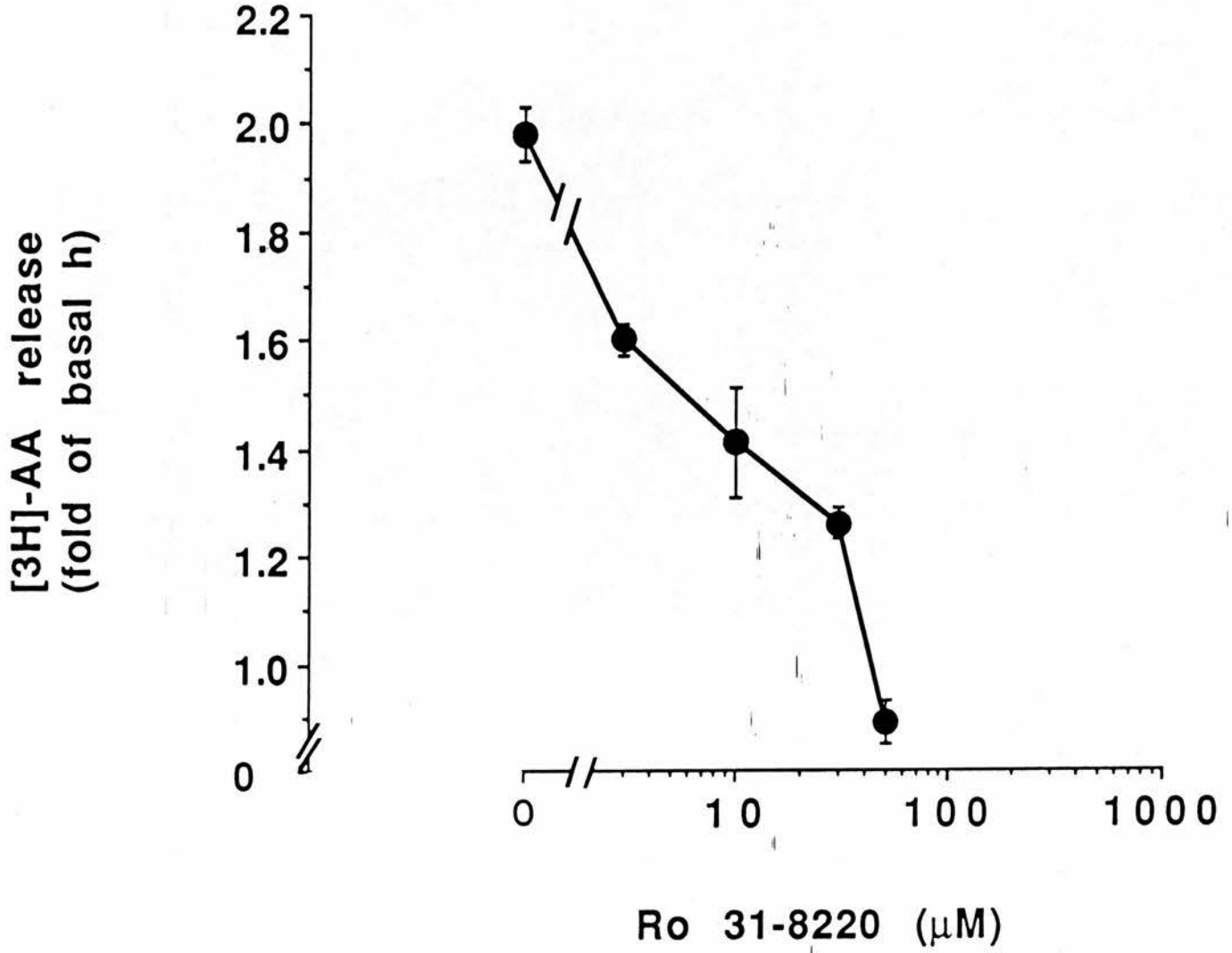

LH release (fold of basal h)
15
10
5
0
1
2
3
*
consecutive hourly incubations

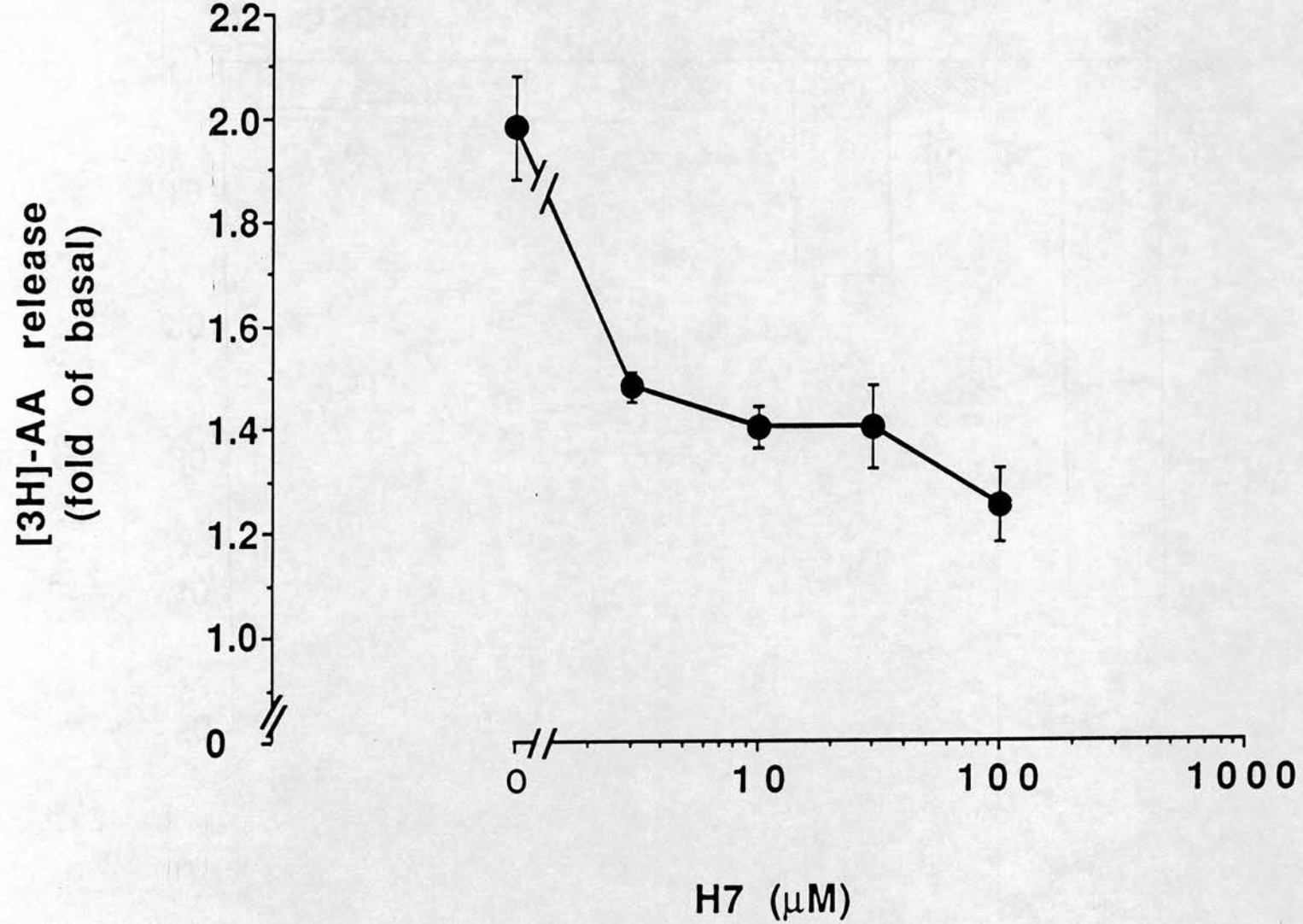

GH release (fold of basal h)
4
3
2
1
0
1
2
3
consecutive hourly incubations


LH release (fold of basal h)
GH release (fold of basal h)
12
10
8
6
4
2
0
4
2
0
0
1
10
100
quinacrine (μM)
*
*

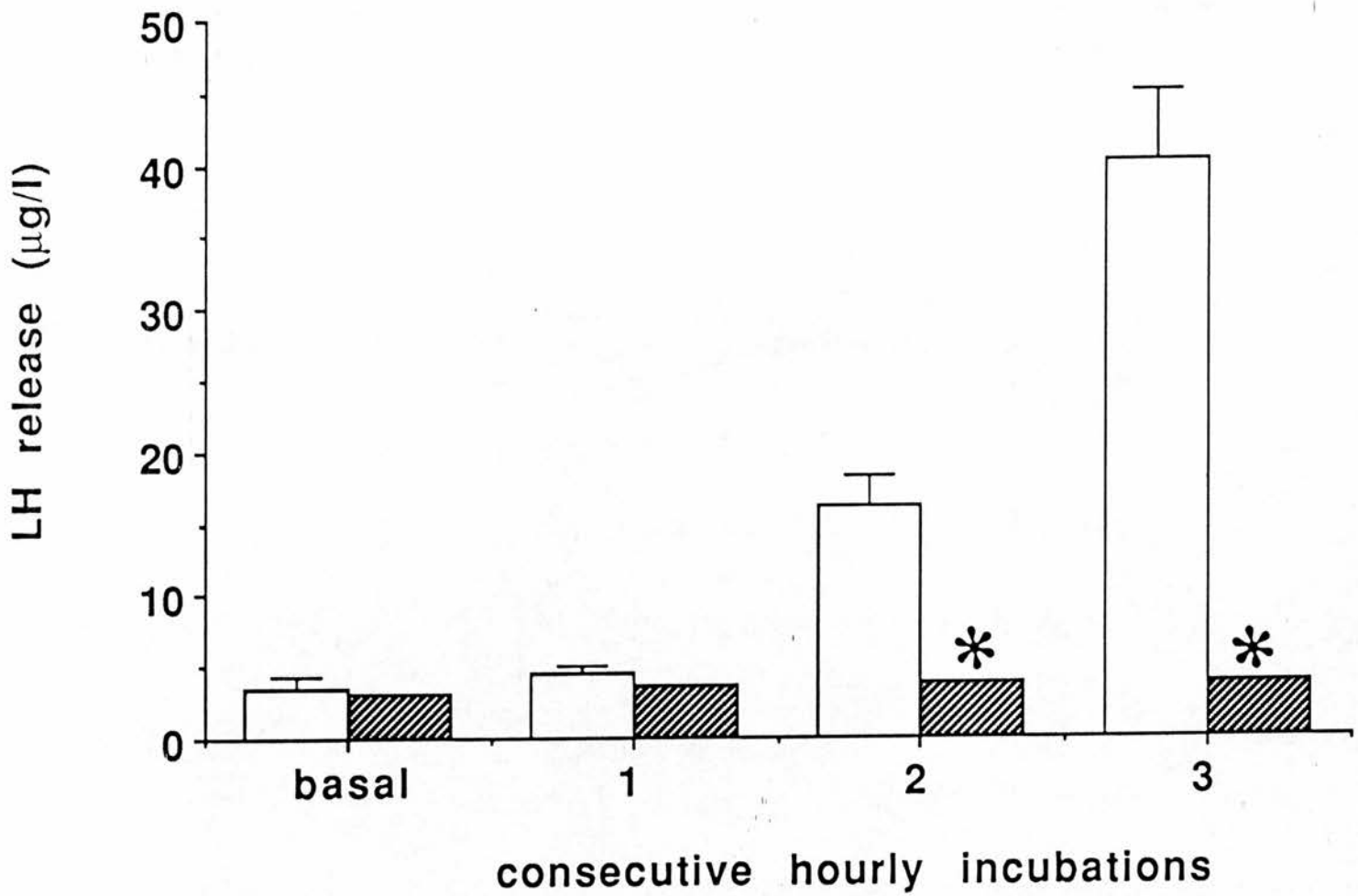

[3H]-AA release
(fold of baseline)
2.0
1.8
1.6
1.4
1.2
1.0
0
*
*
*
0
10
100
1000
PDBu (nM)

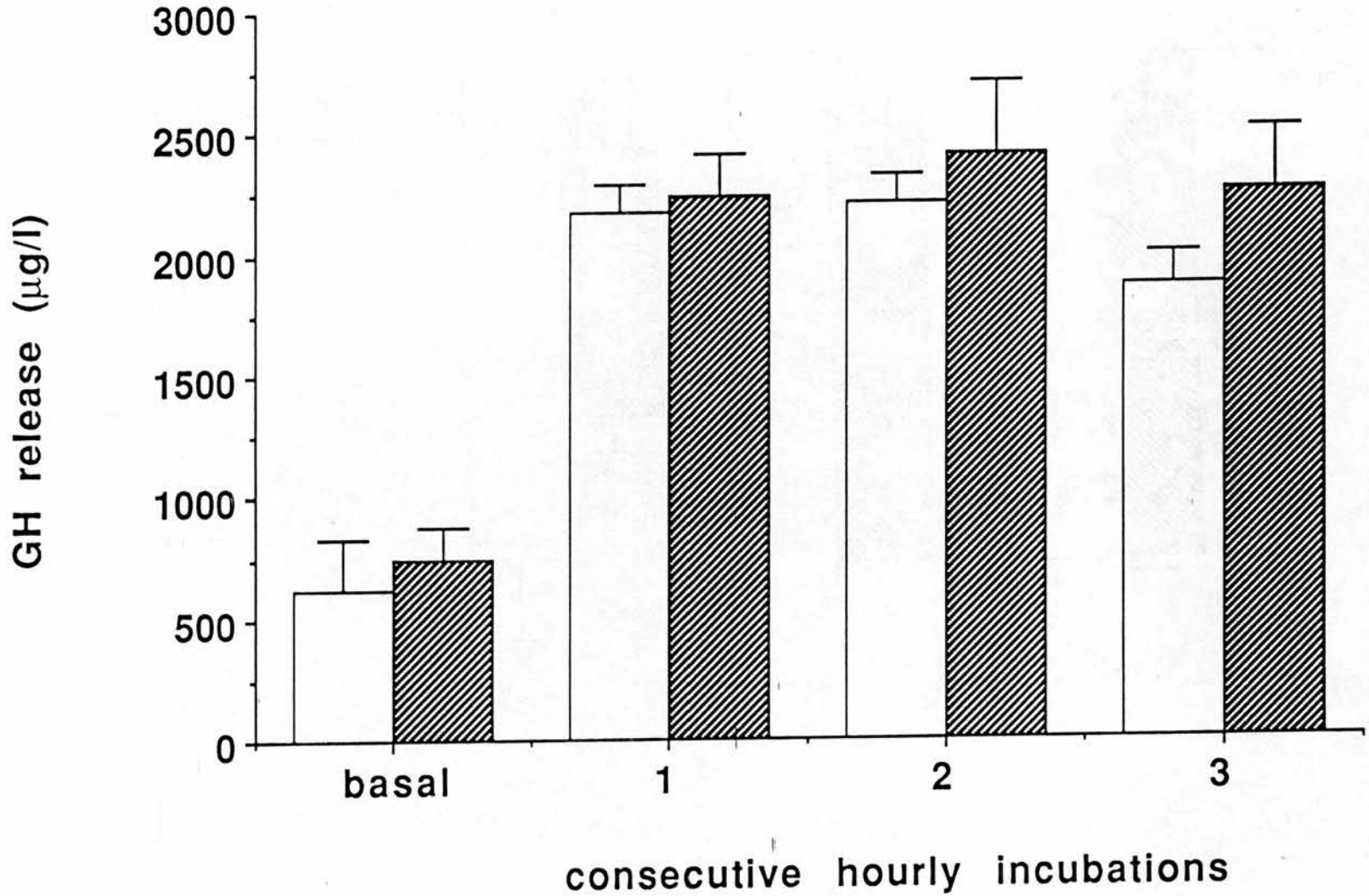

[3H]-AA release
(fold of basal)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0
100
1000
10000
staurosporine (nM)

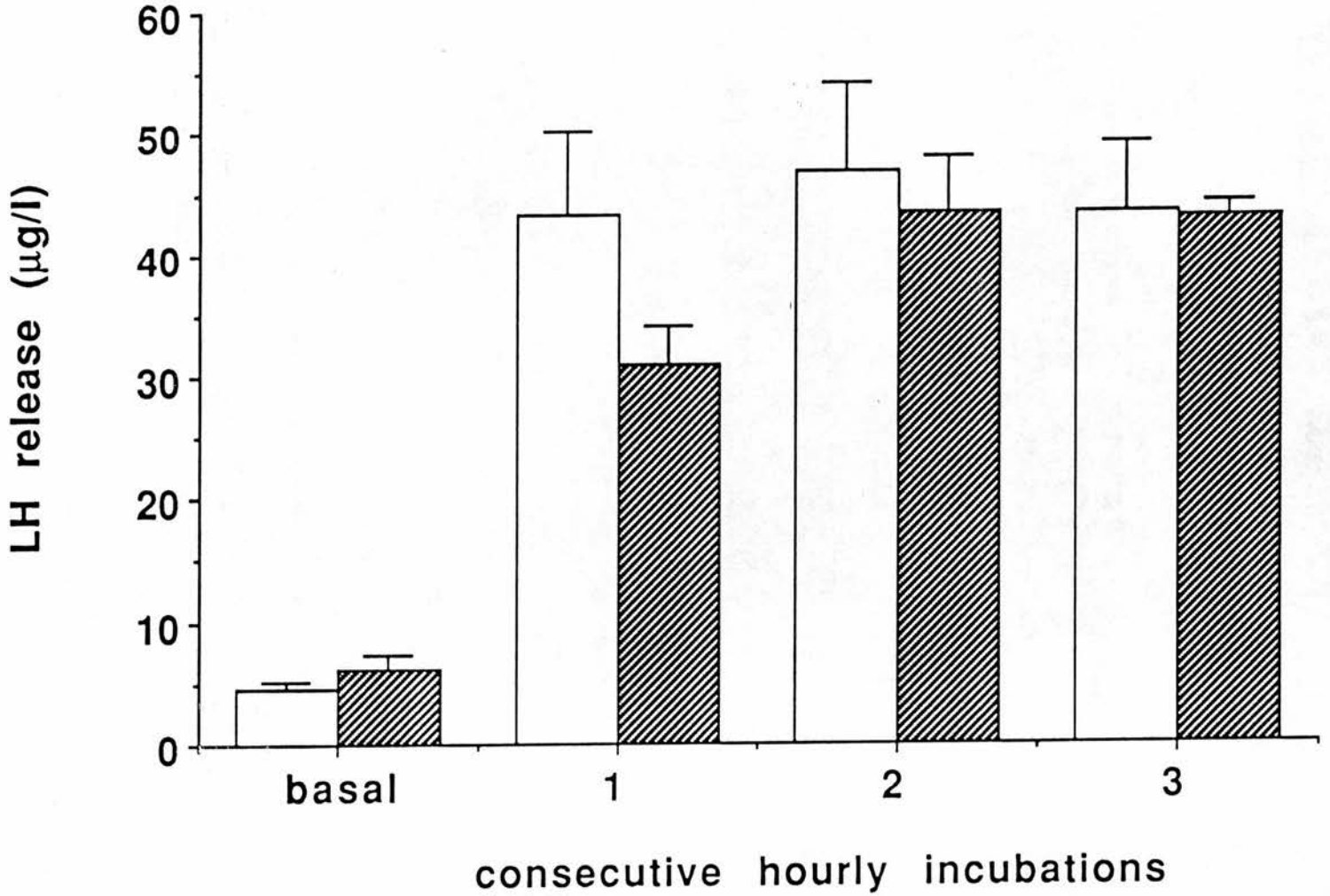

[3H]-AA release
(fold of basal h)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0
10
100
1000
Ro 31-8220 (μM)


[3H]-AA release
(fold of basal)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0
10
100
1000
H7 (μM)

5a


LH release (µg/l)
50
40
30
20
10
0
basal
1
2
3
*
*
consecutive hourly incubations


3000
2500
2000
1500
1000
500
0
GH release (µg/l)
basal
1
2
3
consecutive hourly incubations


LH release (µg/l)
60
50
40
30
20
10
0
basal
1
2
3
consecutive hourly incubations

NEUROSCIENCES ABSTRACT 1991

Cellular actions of pharmacologically distinct forms of Protein Kinase C

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We have developed various cellular models of protein kinase C action which have allowed us to explore, in a physiological context, the selective pharmacology of different species of PKC.

Depolarisation-induced $^{45}Ca^{2+}$ influx into anterior rat pituitary tissue and into GH_3 cells (both of which occur through dihydropyridine-sensitive Ca^{2+} channels) were differently influenced by phorbol esters and putative PKC inhibitors. In GH_3 cells, 4ß-, but not 4α-phorbol 12,13-didecanoate (PDD) inhibited K^+-induced $^{45}Ca^{2+}$ influx in a staurosporine- and H7-sensitive manner. In contrast, PDD enhanced K^+-induced $^{45}Ca^{2+}$ influx into pituitary tissue in a staurosporine-sensitive, but H7-insensitive manner. We have also found certain PKC actions in other models to show differential sensitivity to H7. For example, H7 blocked phorbol 12,13-dibutyrate (PDBu)-induced release of luteinizing hormone (LH) but not growth hormone (GH), whereas staurosporine inhibited both. These results were paralleled by measurements of partially purified PKC activity from anterior pituitary gland. Both Ca^{2+}-independent and Ca^{2+}-dependent PDBu-stimulated kinase activity were blocked with similar potency by staurosporine, whereas Ca^{2+}-independent kinase activity was found to be relatively much more resistant to H7. These pharmacologically different PKCs in anterior pituitary also differ in their cellular targets. Phospholipase A_2 (PLA_2) inhibitors (eg quinacrine) blocked PDBu-induced LH but not GH release suggesting that in gonadotrophes, but not somatotrophes, an H7 sensitive-PKC(s) can act to modulate PLA_2 activity.

This data suggests that PKC species may differ in their sensitivity to PKC inhibitors, and that they may have distinct cellular targets.

PHARMACOLOGICALLY-DISTINCT FORMS OF PROTEIN KINASE C CAN MODULATE RAT ANTERIOR PITUITARY HORMONE RELEASE

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ABSTRACT

To investigate the pharmacology of the protein kinase C (PKC) forms that modulate rat anterior pituitary hormone release, hemipituitaries were incubated in the presence of various PKC activators and inhibitors and luteinizing hormone (LH) and growth hormone (GH) release were measured. Activators of PKC induced LH release with the following order of potency; mezerein > phorbol 12,13-dibutyrate (PDBu) >> 1,2-dioctanoyl-*sn*-glycerol (DOG). Mezerein and PDBu were equipotent, but DOG was ineffective, on GH release. The PKC inhibitor, staurosporine, blocked PKC activator-induced LH and GH release. A component of PDBu- and mezerein-induced LH release was also blocked with high potency by 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H7). However, a second H7-insensitive component was detected. 1,2-Dioctanoyl-*sn*-glycerol-induced LH and mezerein- and PDBu-induced GH release consisted of the H7-resistant component only. These results are consistent with the existence of pharmacologically-distinct PKC forms in rat anterior pituitary tissue.

Key words: protein kinase C, diglycerides, phorbol esters, anterior pituitary hormones

1. INTRODUCTION

The activation of receptors for many different hormones and neurotransmitters is known to result in phosphoinositide hydrolysis and subsequent production of diglycerides and inositol phosphates (Nahorski et al., 1986). Inositol 1,4,5-trisphosphate (Ins 1,4,5 P_3) mobilises intracellular Ca^{2+} from specific stores (Berridge and Irvine, 1984), whilst diglyceride activates the Ca^{2+}/phospholipid dependent protein kinase, protein kinase C (PKC). Both Ca^{2+} mobilisation and PKC activation are important for cell responses to certain hormones (Nishizuka, 1984), including a number that act on anterior pituitary cells.

At least 8 structurally different PKC isoforms (α, β_I, β_{II}, γ, δ, ε, ζ, η) have been identified (Nishizuka, 1988; Parker et al., 1989; Osada et al., 1990). Each PKC form displays somewhat different activation profiles in terms of their requirement for co-factors, such as different phospholipids (Bazzi and Nelsestuen, 1987; Nishizuka, 1988; Parker et al., 1989) and fatty acids (Naor et al., 1988; Murakami and Routtenberg, 1985). Each PKC isoform also differs in its dependence upon Ca^{2+} for activation; the α, β_I, β_{II}, and γ forms (A series) being Ca^{2+}-dependent and the γ, δ, ε, ζ and η isoforms (B series) being Ca^{2+}-independent (Nishizuka, 1988; Huang, 1989; Parker et al., 1989; Osada et al., 1990). Since the PKC isoforms have different patterns of tissue distribution (Brandt et al., 1987; Nishizuka, 1988; Osada et al, 1990), each isoform may have distinct cellular targets and specific physiological roles. Consistent with this hypothesis, studies using cell-free preparations have shown that PKC isoforms can differ in their ability to phosphorylate various artificial and endogenous substrates (Huang, 1989; Parker et al., 1989; Schaap et al., 1989).

We have previously reported that the PKC activator, phorbol 12,13-dibutyrate (PDBu), induces the release of both luteinizing hormone (LH) and growth hormone (GH) from rat anterior pituitary tissue, *in vitro* (Johnson and Mitchell, 1989). However, the temporal patterns of PDBu-induced LH and GH release are quite different, suggesting that distinct intracellular mechanisms and possibly distinct PKC isoforms may be involved in the release of each of these hormones. There is good evidence that PKC is the main cellular receptor for phorbol esters, and that diglycerides interact competitively with the phorbol ester binding domain of PKC (Castagna et al., 1982; Leach et al., 1983; Niedel et al., 1983). However, it is now apparent that PKC isoforms can display different profiles of activation by various species of diglyceride and phorbol ester (Johnson and Mitchell, 1989; Nakadate et al., 1988; Mitchell et al., 1990; MacEwan and Mitchell, 1991; MacEwan et al., 1991; Ryves et al., 1991). We have used our model of anterior pituitary hormone release as a biologically assay of PKC function to examine the possibility that pharmacologically-distinct PKC forms exist in anterior pituitary. The present experiments assessed the effects of various activators and inhibitors of PKC action on LH and GH release from rat anterior pituitary tissue and suggest that a number of pharmacologically-distinct species of PKC are involved in the control of LH and GH release. Some of these data have been previously presented in abstract form (Johnson and Mitchell, 1989).

2. MATERIALS AND METHODS

2.1 Hormone secretion experiments

The methods and experimental design were based on those reported by Pickering and Fink (1976) with the following modifications. Female COB/Wistar rats (Charles River UK Ltd, Margate, Kent), 200-250g body weight, were maintained under controlled lighting (lights on from 0500 to 1900 h) and temperature (22°C) with free access to food pellets (CRM; Labsure, Manea, Cambs, UK) and tap water. Female rats, which had at least two regular 4 day oestrous cycles, were anaesthetised with sodium pentobarbitone (30 mg/kg; Sagatal; May & Baker Ltd, Dagenham, Essex) immediately before being decapitated between 1030 and 1200 h on pro-oestrus. The anterior lobes of the pituitary glands were separated and hemisected. Hemipituitary glands were incubated (one/flask) in 2 ml of Hepes-buffered minimal essential medium with Earles salts (MEM) (Gibco BRL, Paisley, U.K.) at 37°C in a shaking water bath under an atmosphere of 95%O_2/5%CO_2. Hemipituitaries from the same rat were allocated to treatment groups at random. After a preincubation for 30 min the medium was changed for fresh pre-warmed and gassed MEM every hour thereafter. In the initial hour (basal hour), the tissue was incubated with MEM only, followed by consecutive hours (1 h, 2 h, 3 h) in the presence of a PKC activator (PDBu, DOG or mezerein). In the experiments where the effects of PKC inhibitors (staurosporine, H7 or psychosine) were examined, the inhibitor was included throughout all hours of incubation (i.e. basal hour, 1st, 2nd and 3rd hour).

All the media removed from the flasks was stored at -20°C until they were radioimmunoassayed for LH or GH (Niswender et al., 1968).

The standards used were NIH-LH-S18 (Figs 1, 2, 4, 5a and Table 1), NIADDK-rat LH-RP2 (Figs 3, 5b, 5c and Tables 2 and 3) and NIADDK-rat-GH-RP-2.

2.2 Cytosolic PKC activity assay

Anterior pituitary PKC activity was determined as described by Johnson et al., (1992b) using methods modified from those described by Wise et al., (1982) and Huang et al., (1988). Anterior pituitary tissue was homogenised in 2 vol of 20 mM Tris HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 0.01% (w/v) leupeptin and 20 μM E64. The homogenate was centrifuged (16,000 g 20 min, 4°C) and the supernatant was collected and recentrifuged (16,000 g, 5 min, 4°C). The second supernatant was taken to represent cytosol and was partially purified by loading onto 0.5 ml of DE52 diethylaminoethyl cellulose matrix (Whatman International Ltd, Maidstone, Kent, UK) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, C.A., USA) at 4°C. The cytosol was then washed with 6 column vols of homogenisation buffer before eluting the partially-purified PKC with 3 column vols of buffer containing 150 mM NaCl. Partially-purified cytosolic PKC activity was measured in an assay mixture containing (final concentrations): 1.25 mM $MgCl_2$, 100 μg/ml phosphatidyl serine + 0.04% Nonidet P-40, 1.25 mg/ml histone III-S, 100 μM ATP-γ-^{35}S (0.18 μCi/tube) and cytosol. Phosphatidyl serine vesicles were prepared by drying the lipid from chloroform/methanol under a stream of N_2. The subsequent film of phosphatidyl serine was scraped into 20 mM Tris HCl (pH 7.5) + 0.5 mM

EGTA at a stock concentration of 400 μg/ml, sonicated, then 0.16% Nonidet P-40 was added. The mixture was vortexed before use. Assay tubes also contained either 600 μM $CaCl_2$ (100 μM free Ca^{2+}) or 5 mM ethyleneglycol-bis-(β-aminoethyl ether) N, N, N′,N′-tetraacetic acid (EGTA) (less than 3 nM free Ca^{2+}). Protein kinase C activity was measured either in the absence of activator (i.e. basal activity) or in the presence of 1 μM PDBu with or without Ca^{2+} and with or without various concentrations of either H7 or staurosporine. All assay components and drugs were dissolved in 20 mM Tris HCl (pH7.5) + 0.5 mM EGTA. Reactions were carried out at 30°C for 15 min and were stopped by quenching with 20 μl of 0.1 mol/l ATP in 0.1 mol/l EDTA (pH 7.0). The quenched reaction mixture (50 μl) was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd) and washed (3 x 10 ml, 2 min, room temperature) in 75 mM H_3PO_4, dried and counted by liquid scintillation.

2.3 Drugs

Phorbol 12,13-dibutyrate, 1,2-dioctanoyl-*sn*-glycerol, galactosylsphingosine (psychosine) (Sigma Chemical Co Ltd, Poole, Dorset), mezerein and staurosporine (Calbiochem, San Diego, CA, USA) were all made up as stock solutions in dimethylformamide (DMF) and kept in the dark, on ice, before final dilution in MEM for experiments. The maximum concentration of DMF used was 0.5 % v/v and this was shown in control experiments to have no effect. 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H7) (Gibco BRL, Paisley, Scotland, UK), leupeptin (Boehringer Mannheim, Lewes, UK) and trans-

epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64, Sigma) were made up as stock solutions in distilled H_2O. Phenylmethlysulphonyl fluoride (PMSF) and histone III S were obtained from Sigma, Nonidet P-40 and unlabelled ATP-γ-S were from Calbiochem. Phosphatidyl serine (sodium salt) was from Lipid Products, Nutfield, Surrey and ATP-γ-^{35}S (specific activity = 1256 Ci/mmol) was purchased from DuPont, Dreiech, Germany. Analar grade laboratory chemicals were obtained from BDH, Dagenham, Essex, UK.

2.4 Statistics

Data are expressed as means ± standard error of the mean (S.E.M). Statistical analyses were carried out using the Mann Whitney U-test. The EC_{50} values for the PKC activators and the IC_{50} values for the kinase inhibitors were obtained from fitting the data with the non-linear, error-weighted iterative curve fitting programme, P.fit (Biosoft, Cambridge, UK) and are given as mean ± S.E.M.

3. RESULTS

3.1 The effect of PKC activators on anterior pituitary hormone release

When rat hemipituitary pieces were incubated for consecutive hours in the presence of PDBu (100 nM), both LH and GH release significantly increased above basal h levels. However, the time course of PDBu-induced release was very different for each hormone (Fig 1). The release of GH was maximal during the 1st h of PDBu incubation, with release increasing by 2.1 ± 0.3 fold over basal h levels. In contrast, LH release in the 1st h of incubation with PDBu was not significantly different from LH release measured during the basal h. During the 2nd

h of incubation with PDBu, LH release increased to 2.9 ± 1.0 fold of basal h levels ($p < 0.05$) and increased further in the 3rd h of incubation (5.3 ± 0.9 fold of basal h levels, $p < 0.01$).

Another PKC activator, mezerein, induced both LH and GH release from hemipituitary pieces with temporal patterns similar to those observed using PDBu (data not shown). Increasing the concentration of either PDBu or mezerein resulted in a concentration-dependent increase in the magnitude of both LH (Fig 2a) and GH (Fig 2b) release. Growth hormone release was induced by mezerein and PDBu with similar potency (EC_{50} of 74 ± 62 nM and 82 ± 25 nM respectively). Mezerein was almost as potent in releasing LH (EC_{50} = 152 ± 70 nM) . However, much greater concentrations of PDBu were required to elicit LH release, with 50% of the maximal release induced by mezerein only being reached by a PDBu concentration of approximately 900 nM. It appears, therefore, that PDBu is considerably less potent than mezerein at inducing LH release. These results suggest that the PKC(s) which are involved in LH release may be less susceptible to activation by PDBu than mezerein.

The cell-permeable diglyceride, DOG (200 μM) induced a small but significant increase in LH secretion from rat anterior pituitary tissue throughout the 2nd h and 3rd h of incubation with the diglyceride ($p < 0.05$) (Fig 3a). However, even at this high concentration, DOG was less effective at inducing LH release when compared to either PDBu or mezerein. Growth hormone release was not significantly increased above baseline levels at any hour of incubation with DOG (Fig 3b)

suggesting that the PKC(s) which induce GH release may be relatively resistant to activation by DOG.

3.2 The effect of PKC inhibitors on anterior pituitary hormone release

The PKC inhibitor, staurosporine, readily blocked 100 nM PDBu-induced release of both LH and GH with similar potency (IC_{50} = 44 ± 17 nM and 54 ± 42 nM for LH and GH respectively) (Fig 4). At staurosporine concentrations of 100 nM and above, both LH and GH release were blocked to levels which were not significantly different from basal h levels of release.

The effects of another putative endogenous PKC inhibitor, psychosine (galactosylsphingosine) (Hannun and Bell, 1987), was examined on PDBu-induced LH and GH release (Table 1). When anterior pituitary tissue was incubated with psychosine alone, both LH and GH were released to levels significantly higher than baseline ($p < 0.05$). Psychosine had no inhibitory action on either PDBu-induced LH or GH release; in fact , PDBu-induced release of LH and GH were both slightly enhanced in the presence of psychosine, although not to significant levels, presumably indicating non-specific actions of this substance.

Luteinizing hormone release induced by 100 nM PDBu was significantly inhibited by another PKC inhibitor, H7, at concentrations of 3 μM and above and with an IC_{50} value of 1.7 ± 1.5 μM (Fig 5a). In contrast, 100 nM PDBu-induced GH release was unaffected by H7, even at concentrations of 30 μM and above which caused over 80 % inhibition of LH release induced by 100 nM PDBu. However, when the effect of H7 (30

μM) was examined on LH release measured over a range of PDBu concentrations (Fig 5b), it was clear that an H7-resistant component of this response could also be detected at higher levels of the phorbol ester. That is, LH release induced by 30 nM PDBu was completely inhibited by 30 μM H7, whereas, approximately 20 % of LH release measured at 1 μM PDBu was insensitive to H7 (30 μM). Similarly, mezerein (300 nM)-induced LH release also consisted of H7-sensitive and resistant components. Approximately 40 % of the LH secretory response to mezerein was blocked by H7 at concentrations as low as 1 μM. However, further inhibition of the response was not seen until H7 concentrations of 30 μM. Furthermore, at H7 concentrations as high as 100 μM, mezerein-induced LH release was not completely inhibited, with 20 % of the response remaining. Mezerein (300 nM)-induced GH release was completely insensitive to inhibition by H7 at concentrations up to 100 μM (Fig 5c). Both mezerein-induced LH and GH release were, however, readily blocked by 300 nM staurosporine (Table 2).

Although 200 μM DOG-induced LH release was readily inhibited by staurosporine (300 nM) to levels that were not significantly different from baseline, this response was entirely resistant to block by 30 μM H7 (Table 3). Therefore, DOG may be a selective activator of the H7-resistant PKC(s) which can elicit LH release.

Neither H7 (30 μM) nor staurosporine (2 μM) had any effect on baseline LH and GH release from anterior pituitary tissue when added alone to the incubation medium over a period of 3 consecutive hours (data not shown).

Anterior pituitary PKC activity, measured in a mixed micelle assay, was used to further examine the effects of H7 and staurosporine. Calcium-independent, cytosolic PKC activity was stimulated by PDBu with an EC_{50} of 916 ± 150 nM and the additional activity evoked by addition of Ca^{2+} had an EC_{50} for PDBu of 261 ± 86 nM. All activity was entirely dependent on the presence of phosphatidyl serine. Calcium-dependent activity induced by 1 μM PDBu was potently inhibited by staurosporine and H7 (Table 4) with IC_{50} values similar to those found for PKC activity measured in a range of other peripheral and central tissues. For example, Ca^{2+}-dependent cytosolic PKC activity from rat midbrain was inhibited by staurosporine and H7 with IC_{50} values of 100 ± 40 nM and 34 ± 5 μM respectively. In contrast, Ca^{2+}-independent anterior pituitary PKC activity was distinctly less sensitive to H7 than the Ca^{2+}-dependent activity measured in the same experiment (Table 4). However, in experiments using rat midbrain, Ca^{2+}-independent activity had the expected sensitivity to H7 and staurosporine (IC_{50} = 27 ± 9 μM and 120 ± 6 nM respectively). The Ca^{2+}-independent activity in anterior pituitary, however, showed the expected potency of block by staurosporine (Table 4).

4. DISCUSSION

Several earlier reports have shown that PKC activation by phorbol esters and diglycerides can induce hormone release from anterior pituitary tissue, *in vitro*, and from anterior pituitary cells in culture (Smith and Vale, 1980; Conn et al., 1985; Nigro-Villar and Lapetina, 1985; Turgeon and Waring, 1986; Johnson and Mitchell, 1989). However, we

have shown that LH release is induced more effectively by mezerein than PDBu, suggesting that the PKC(s) involved are more readily activated by mezerein. In contrast to their effects on LH release, PDBu and mezerein induced GH release with equal potency, suggesting that the PKC(s) that are involved in the latter case are activated by these two compounds with equal effectiveness. Differences in the tumour-promoting effects of phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) ,and mezerein have been reported previously (Slaga et al., 1980) and may also reflect the ability of these compounds to preferentially activate certain forms of PKC.

1, 2-Dioctanoyl-*sn*-glycerol was unable to induce any significant release of GH suggesting that DOG-insensitive PKC(s) are involved in this response. Although DOG could enhance LH release, the secretory response to DOG was much smaller than that measured using either PDBu or mezerein. In certain other models of PKC function in pituitary cells, for example, the facilitation of depolarisation-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms (MacEwan and Mitchell, 1991), a DOG concentration of 100 μM elicited effects of equivalent magnitude to 300 nM PDBu or 300 nM mezerein. However, the lower potency of DOG on LH release may reflect the ability of this diglyceride to activate only a sub-population of the PKCs that are potentially involved in LH release. Consistent with this hypothesis, DOG appeared to be a selective activator of an H7-resistant PKC(s) involved in LH release. In other physiological models of PKC action, DOG has been shown to mimic some, but not all, actions of phorbol esters (Lacerda et al., 1988; MacEwan and Mitchell, 1991; Thomson et al., 1992). Indeed, in both the hormone release and

$^{45}Ca^{2+}$ flux models, DOG appears to selectively activate a relatively H7-resistant PKC, which is also activated by PDBu (MacEwan and Mitchell, 1991; Thomson et al., 1992), further suggesting that DOG may be a selective activator of certain PKC forms. Reduced potency of DOG as an activator of the α-PKC isoform has been reported (Sekiguchi et al., 1988) and is most marked under conditions of basal cytosolic Ca^{2+} (≤ 200 nM). However, DOG, unlike PDBu and mezerein, is susceptible to intracellular degradation (Kaibuchi et al., 1983), which may also partly contribute to the low potency of DOG on LH release, especially under the relatively long incubation periods used here.

We have demonstrated here that some actions of PKC activators on anterior pituitary hormone release are unusually resistant to the PKC inhibitor, H7 (Hidaka & Hagiwara, 1987), but not to staurosporine (Tamaoki et al., 1986). Both H7-sensitive and -insensitive PKC(s) may be involved in PKC activator-induced LH release, with DOG being a selective activator of the H7-resistant PKC(s). In contrast, the PKC(s) which induce GH release are of the H7-resistant type only but are not activated by DOG. These observations suggest that at least 3 forms of PKC (or a closely related kinase) with distinct pharmacological properties may participate in phorbol ester-induced anterior pituitary hormone release. Additional evidence that H7-resistant PKC forms are expressed in anterior pituitary tissue has come from cell free assays of PKC activity. In these experiments, a PDBu-induced, Ca^{2+}-independent, phosphatidyl serine-dependent component was detected which was relatively H7 resistant but readily blocked by staurosporine. In contrast, Ca^{2+}-dependent, PDBu-induced activity displayed relative sensitivity to block

by H7 and staurosporine. We and others have previously described phorbol ester-responses which show unusual resistance to H7, but not staurosporine (Watson et al., 1988; Johnson and Mitchell, 1989; Johnson et al., 1989; Nakadate et al., 1989; Fink et al., 1990; Johnson et al., 1992a; Thomson et al., 1992). Furthermore, an H7-resistant PKC appears to have a role in anterior pituitary responses to LH-releasing hormone (Fink et al., 1990; Johnson et al., 1992a).

Although staurosporine and H7 are structurally very different, they both appear to block PKC activity by binding at or near to the ATP binding site (Huang, 1989), hence the contrast between the inhibitory effects of H7 and staurosporine may at first be unexpected. However, these two inhibitors may not have identical sites of action. Both H7 and staurosporine have been reported to exert kinetically competitive inhibition with respect to ATP (Hidaka et al., 1984; Tamaoki et al., 1986; Rüegg and Burgess, 1989) although, in both cases, the precise site of action is unclear. However, the binding of [^{3}H]N,N-dimethylstaurosporine (a radiolabelled staurosporine derivative) is displaced by H7 with extremely low potency (IC_{50} > 500 μM) (Thomson et al., 1991a). In addition, the α and β PKC isoforms have both been reported to have a second consensus recognition motif for ATP (Huang, 1989; Burns and Bell, 1991), therefore the interactions between H7 and these isoforms may be complex. Since H7 and its congener, HA 1004, can display relative selectivity for particular kinases (Hidaka and Hagiwara, 1987), it may be possible that these compounds can have some degree of selectivity between different PKC isoforms.

The different temporal patterns of PKC-activator induced LH and GH release indicates that these pharmacologically-distinct PKCs may also phosphorylate distinct cellular targets. For example, evidence from our laboratory suggests that these distinct PKCs may differentially modulate voltage-sensitive Ca^{2+} channels (MacEwan et al., 1991) and phospholipase A_2 (Thomson et al., 1991b). In the pituitary derived GH_4C_1 cell line, a specific role has been attributed to the ε isoform in cellular responses to thyrotropin releasing hormone (Kiley et al., 1990, 1991) further indicating that specific PKC isoforms have distinct roles in anterior pituitary cell function.

The relationship of the PKC forms involved here in LH and GH release to the known PKC isoforms is unclear. Of the known PKC types, α, β, ε, but not γ, have been described in anterior pituitary cells (Naor et al., 1988; Kiley et al., 1990, 1991; Naor, 1990). Our own experiments, employing Northern blotting with 3' tailed oligonucleotide probes for particular isoforms, indicate that the ζ and δ isoforms may also be expressed in anterior pituitary tissue (data not shown). The η isoform is reported to show a broad tissue distribution (Osada et al., 1990), so it too may potentially be a mediator of some of the effects seen here. When added to permeabilized pituitary cells, both α and β isoforms can stimulate LH release (Naor et al., 1989). Certainly, the results presented here indicate that the H7-sensitive PKC(s) which are involved in LH release may represent one or more of the Ca^{2+}-dependent types, such as the α and β isoforms. However, PKC-induced LH release and GH release also involve H7-resistant PKC(s) which appear to be Ca^{2+}-independent, and may therefore represent one or more of the δ, ε, ζ and η isoforms.

Since data obtained using recombinant or purified enzyme preparations has shown that the ε- and δ-isoforms of PKC display the same degree of sensitivity to H7 (Schaap and Parker, 1990; Uchida et al., 1991), the Ca^{2+}-independent, H7-resistant PKC(s) that induce LH and GH in our model may not represent either of these isoforms. However, there is increasing evidence that the degree of Ca^{2+}-dependency of the α, β and γ isoforms, may alter in the presence of certain fatty acids (Naor et al., 1988; Shinomura et al., 1991) and it has also been suggested that the phosphorylation state of the β isoform can alter its Ca^{2+} dependency (Pelech et al., 1991). Thus, it is possible that a modified state of one or more of the A series PKC isoforms may represent one of the apparently Ca^{2+}-independent, H7-resistant types which control anterior pituitary hormone release.

In summary, we have shown here that several pharmacologically distinct forms of kinase, with many of the properties of PKC, exist in anterior pituitary tissue and these forms differ in their influence on hormone release from different cell types. The relationship of these pharmacologically distinct forms to the known PKC isoforms, is unclear.

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Table 1. Effect of psychosine on PDBu-induced LH and GH release from rat anterior pituitary tissue. Hemipituitaries were incubated for a basal hr with either no drug (baseline) or psychosine (50 μM), followed by 3 consecutive hrs with the same or, in addition, PDBu (100 nM). Values shown here are for GH release measured in the 1st h and LH release measured in the 3rd h of treatment. Values are mean ± S.E.M., and the number of determinations are in parentheses.

	LH release (ng/ml)	GH release (ng/ml)
baseline	2.5 ± 0.3 (6)	464 ±70 (5)
psychosine	22.7 ± 4.1 (6)	1424 ± 109 (6)
PDBu	33.8 ± 8.3 (7)	1951 ± 216 (7)
psychosine + PDBu	60.1 ± 11.7 (4)	2243 ± 226 (4)

Table 2. Effect of staurosporine on mezerein-induced LH and GH release from rat anterior pituitary tissue. Hemipituitaries were incubated for a basal hr with either no drug (baseline) or staurosporine (300 nM), followed by 3 consecutive hrs with the same and/or mezerein (300 nM). Values shown here are for GH release measured in the 1st h and LH release measured in the 3rd h of incubation. Values are mean ± S.E.M., with the number of determinations shown in parentheses.

	LH release (ng/ml)	GH release (ng/ml)
baseline	3.9 ± 0.4 (4)	480.7 ± 105.5 (4)
mezerein	39.1 ± 4.3 (5)	1820.3 ± 234.7 (4)
mezerein + staurosporine	*2.9 ± 0.4 (4)	*510.0 ± 38.7 (4)

Table 3. The effect of PKC inhibitors on DOG-induced LH release from rat anterior pituitary tissue. Hemipituitaries were incubated for a basal h in the absence of drugs (baseline) or with H7 (30 μM) or staurosporine (300 nM) followed by consecutive hours, in addition, with DOG (200 μM). Values shown here are for LH release measured in the 3rd h of incubation. Staurosporine, but not H7, significantly inhibited DOG-induced LH release (*$p < 0.05$). Data are means ± S.E.M. and the number of determinations are shown in parentheses.

	LH release (ng/ml)
baseline	3.6 ± 0.3 (5)
DOG	8.5 ± 0.8 (6)
DOG + staurosporine	*2.4 ± 0.4 (6)
DOG + H7	7.7 ± 0.3 (6)

Table 4. Effect of PKC inhibitors on PDBu-induced PKC activity partially purified from rat anterior pituitary cytosol. Phorbol 12,13-dibutyrate (1 μM)-induced histone IIIs kinase activity was measured in the presence of phosphatidyl serine, with either the presence (100 μM free Ca^{2+}) or absence (> 3 nM free Ca^{2+}) of Ca^{2+} as described in section 2.2.

	IC_{50} values	
	Staurosporine (nM)	H7 (μM)
Ca^{2+}-dependent	101 ± 39	17 ± 4
Ca^{2+}-independent	171 ± 46	121 ± 18

Figure 1

Temporal profile of the effect of PDBu on LH and GH release from rat hemipituitaries. Tissue was incubated for 4 consecutive hours, initially with no drug (basal h), followed by 3 consecutive hours (1st h, 2nd h, 3rd h) in the presence of PDBu (100 nM) and LH (open bars) and GH (hatched bars) release were determined. The release of GH was maximal by the 1st h of PDBu incubation whereas LH release was only significantly greater than basal h levels in the 2nd h and 3rd h of PDBu incubation ($p > 0.05$). Data are means ± S.E.M. for 5 - 11 determinations.

Figure 2

Dose response curves for the effect of (■) PDBu and (●) mezerein, on the release of LH (a) and GH (b) from rat anterior pituitary tissue. Hemipituitaries were incubated for an initial basal h with no drug followed by consecutive hours in the presence of various concentrations of PDBu or mezerein. These graphs show LH release measured in the 3rd h and GH release measured in the 1st h of incubation with these compounds. Data are expressed as the fold of release that occurred in the initial basal h. Each point represents the mean ± S.E.M. for 4 - 11 determinations.

Figure 3

The temporal pattern of DOG-induced (a) LH and (b) GH release from rat hemipituitary pieces. Tissue was incubated for 4 consecutive hours in the presence of medium only (baseline, open bars) or for an initial, basal h in medium only followed by consecutive hs with 200 μM DOG

(hatched bars). The release of LH was significantly greater than baseline levels throughout the 2nd and 3rd h of DOG incubation ($p < 0.05$). However, GH release was not significantly different from baseline levels during any hour of incubation with DOG. Data are means ± S.E.M. for 4 - 6 determinations.

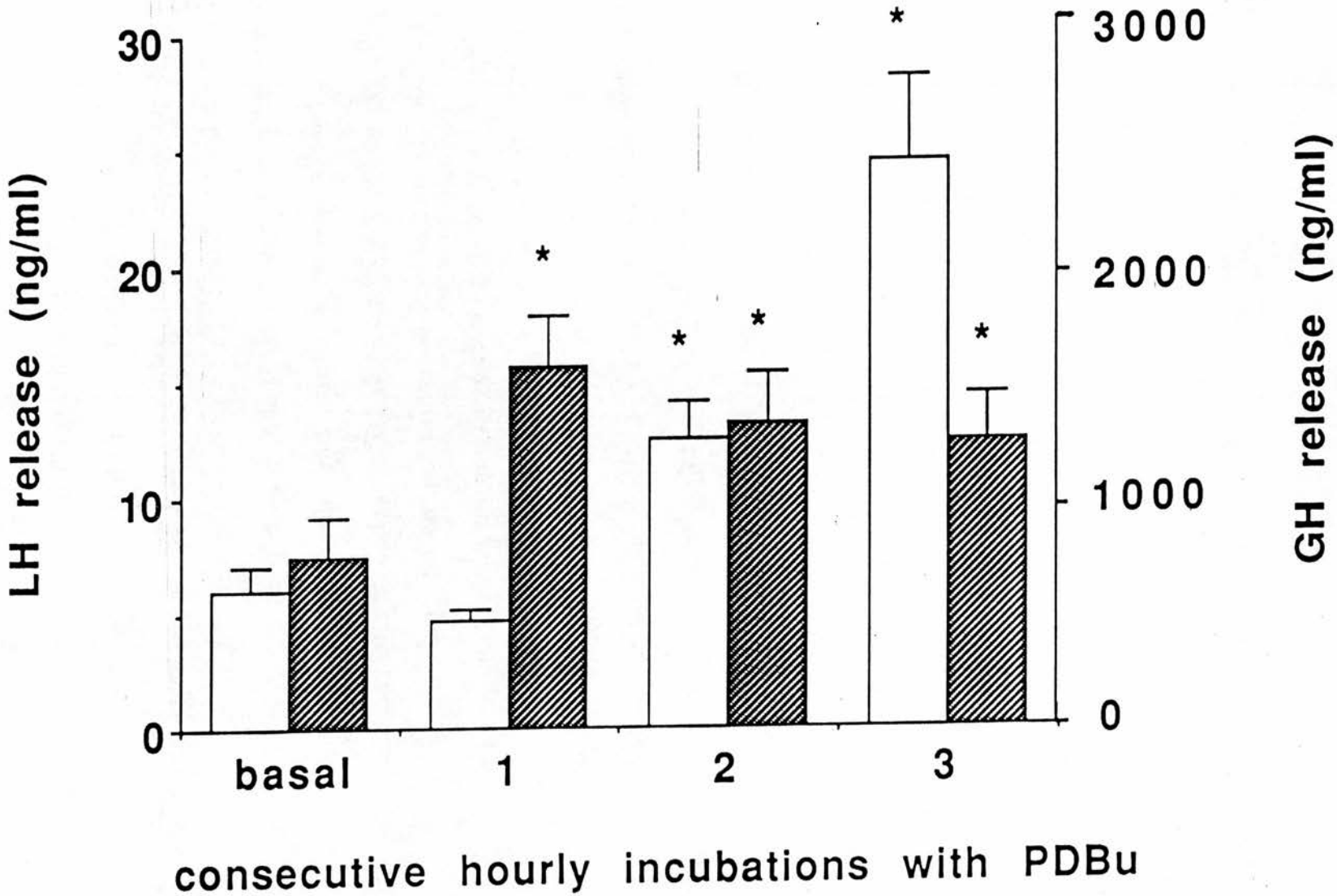
Figure 4

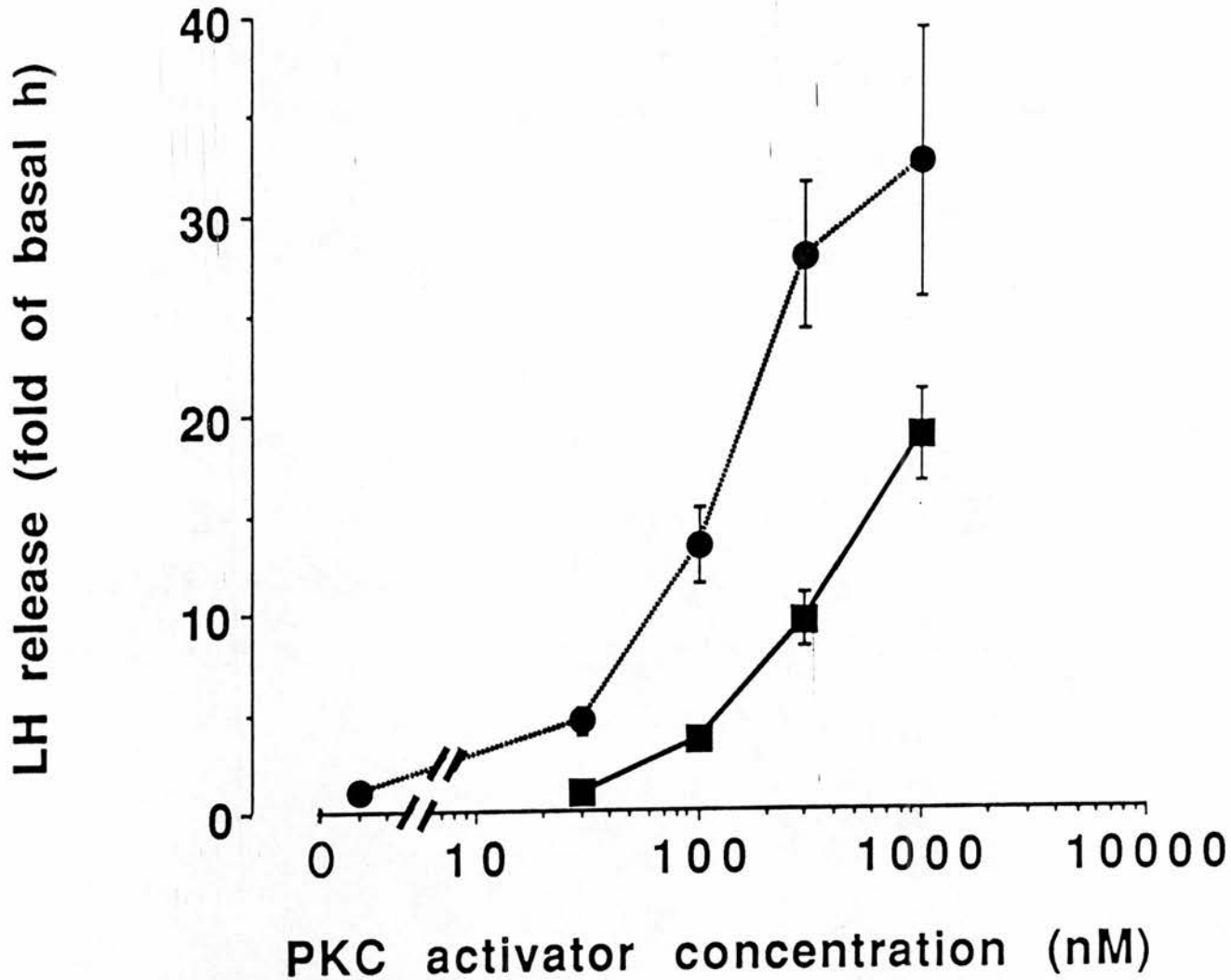
Concentration-response curves for the effect of the PKC inhibitor, staurosporine, on PDBu-induced LH (●) and GH (■) release from rat anterior pituitary tissue. Tissue was incubated over consecutive hours (basal h, 1st h, 2nd h, 3rd h) in the presence of staurosporine (10 - 300 nM). In addition, PDBu (100 nM) was also present throughout the 1st h, 2nd h and 3rd h incubations. The data shows the effects of staurosporine on 3rd h PDBu-induced LH release and 1st h PDBu-induced GH release. Staurosporine significantly inhibited ($p < 0.05$) both PDBu (100 nM)-induced LH and GH release with similar potency. Each point on the curves represents the mean ± S.E.M. for 4 - 6 determinations.

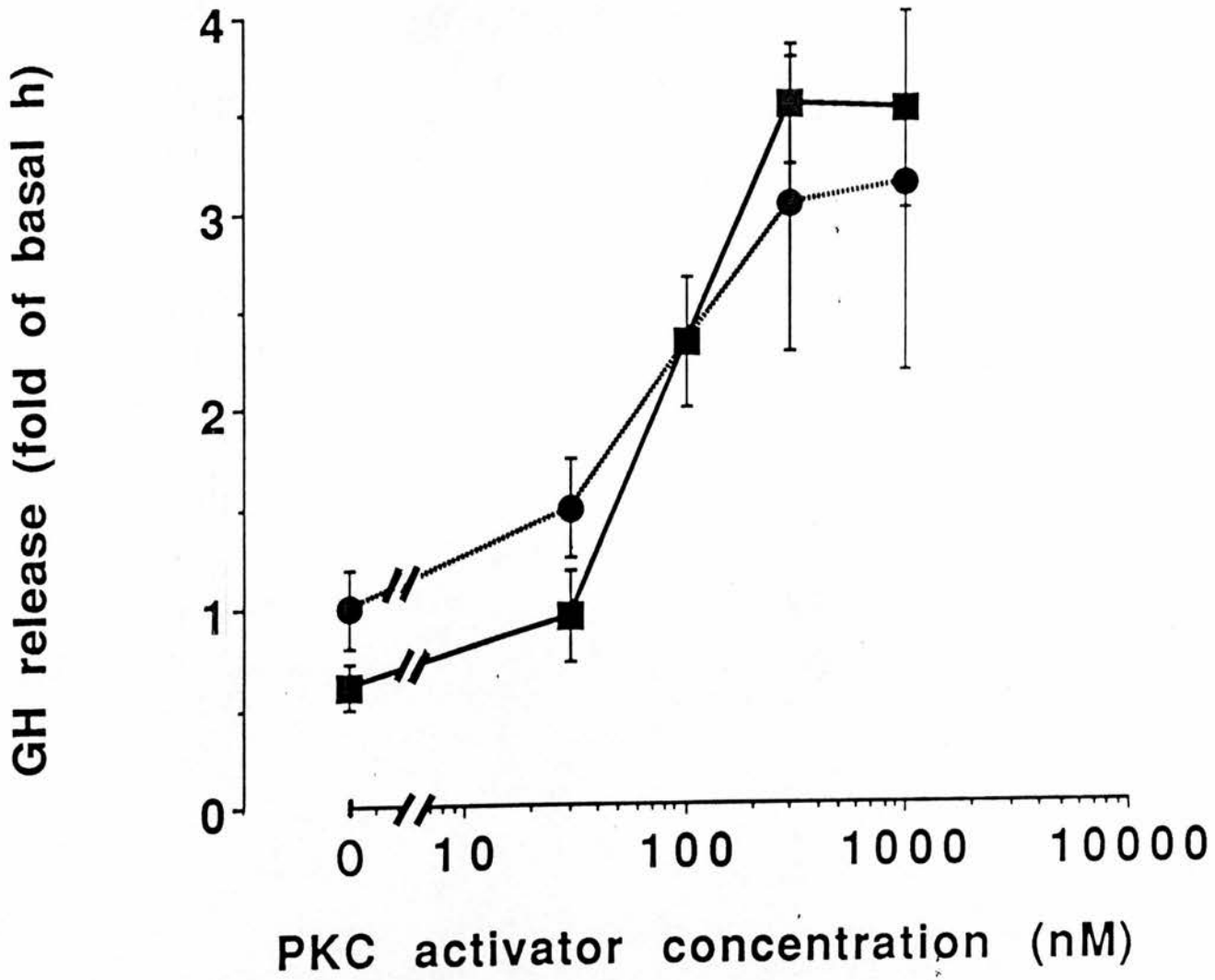
Figure 5

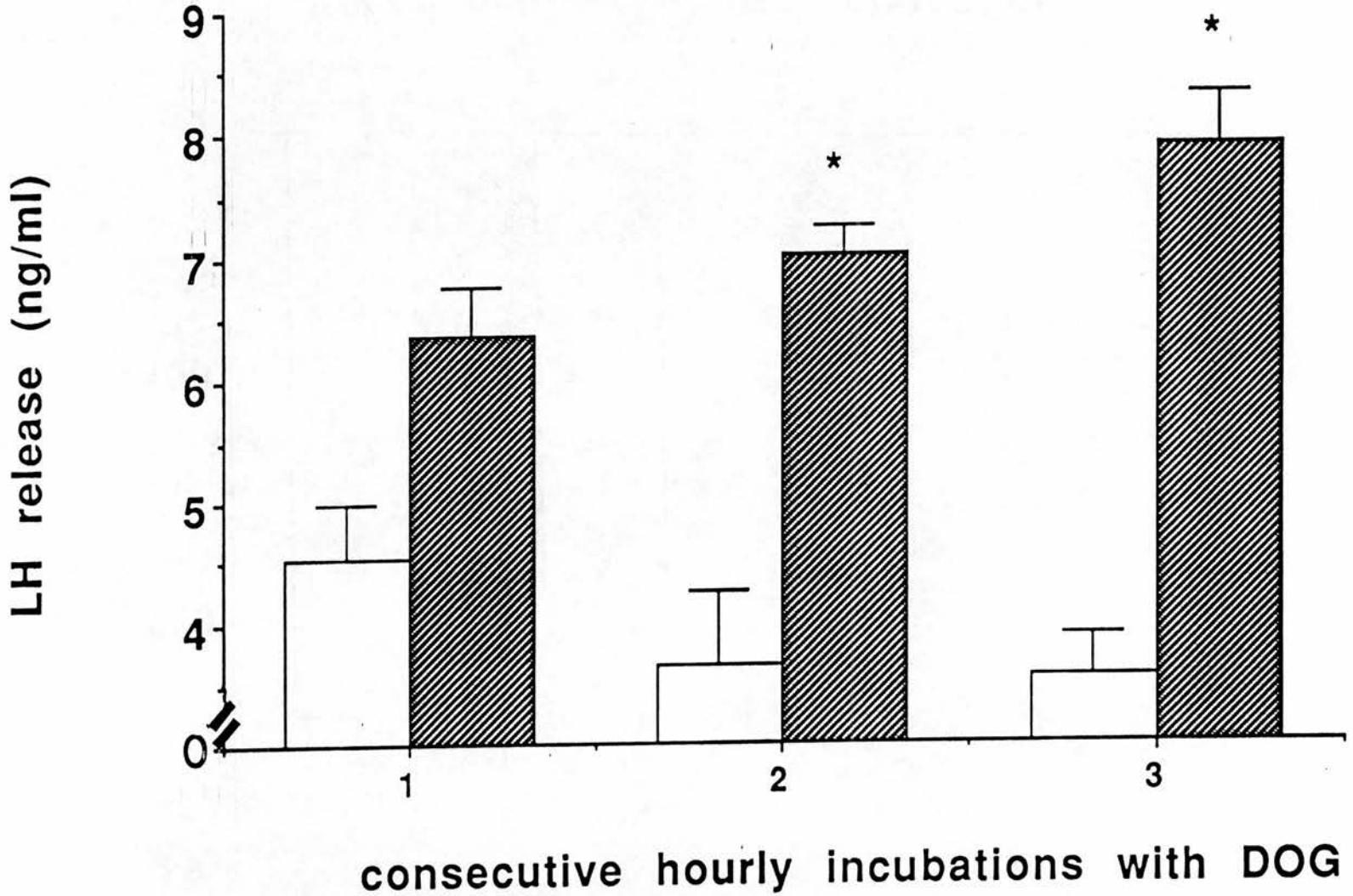
Concentration-response curves for the effect of (a) H7 on 100 nM PDBu-induced LH and GH release (b) H7 (30 μM) on LH release induced by increasing PDBu concentrations and (c) H7 on mezerein-induced LH and GH release from rat anterior pituitary tissue. (●) LH release; (■) GH release. In parts (a) and (c), tissue was incubated for consecutive hours (basal h, 1st h, 2nd h, 3rd h) in the presence of various concentrations of

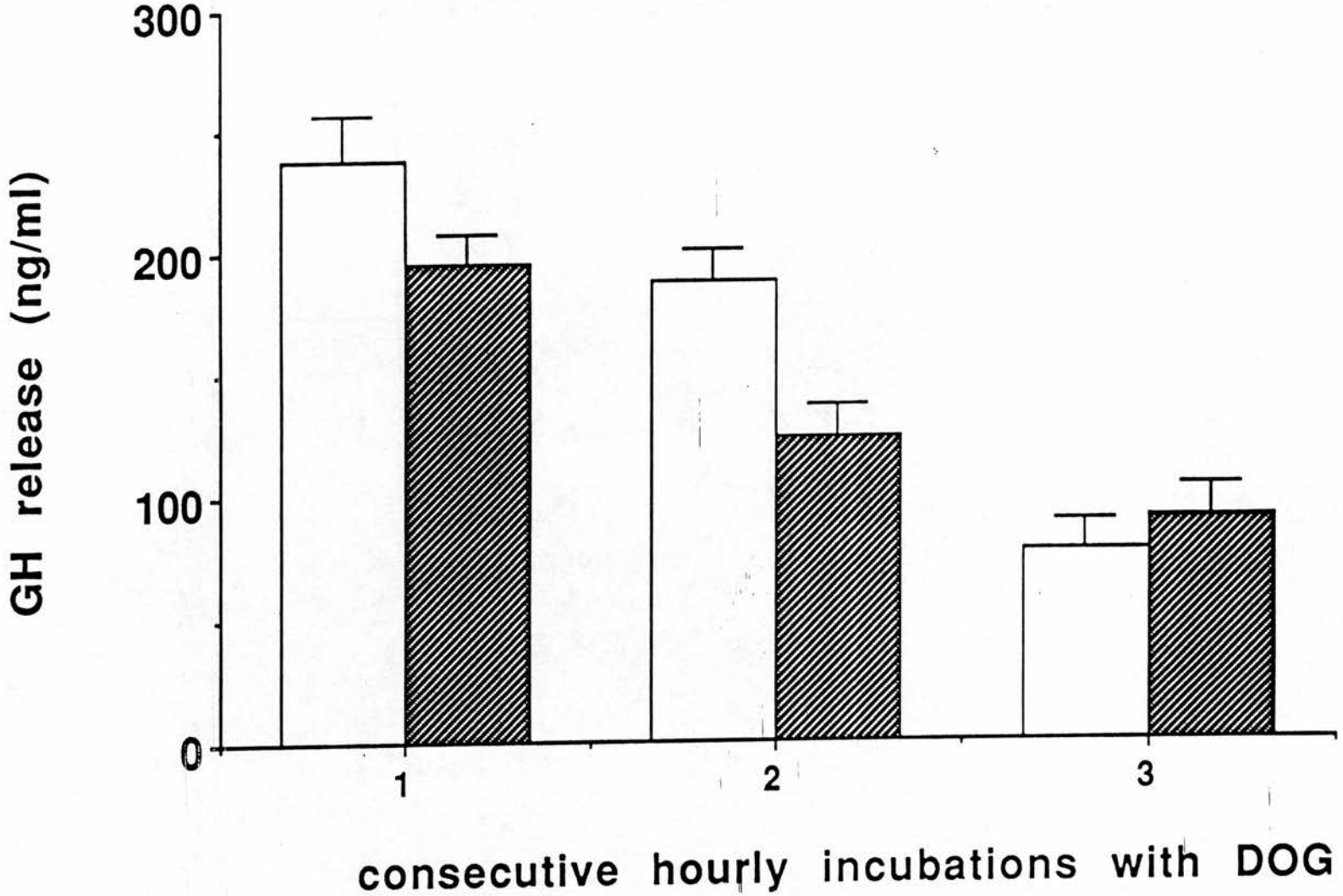
H7. In addition, either 100 nM PDBu (a) or 300 nM mezerein (c) was also present throughout the 1st h, 2nd h and 3rd h incubations. The data show GH release measured during the 1st h and LH release measured in the 3rd h of incubation with PKC activators. Statistical significant inhibition of hormone release by H7 is shown by * ($p < 0.05$). The corresponding basal release of LH during the 3rd h of drug-free controls was 2.5 ± 0.3 ng/ml (a) and 3.9 ± 0.4 (c). H7 alone had no effect on basal release of LH or GH at any of the concentrations tested. In part (b), tissue was incubated for consecutive hours with various concentrations of PDBu (3 - 1000 nM) either in the presence or absence of H7 (30 μM). The data shows the % of the LH response, measured during the 3rd h of PDBu incubation, that was inhibited by H7. Each point on the graphs represents the mean ± S.E.M. for 4 - 6 determinations.

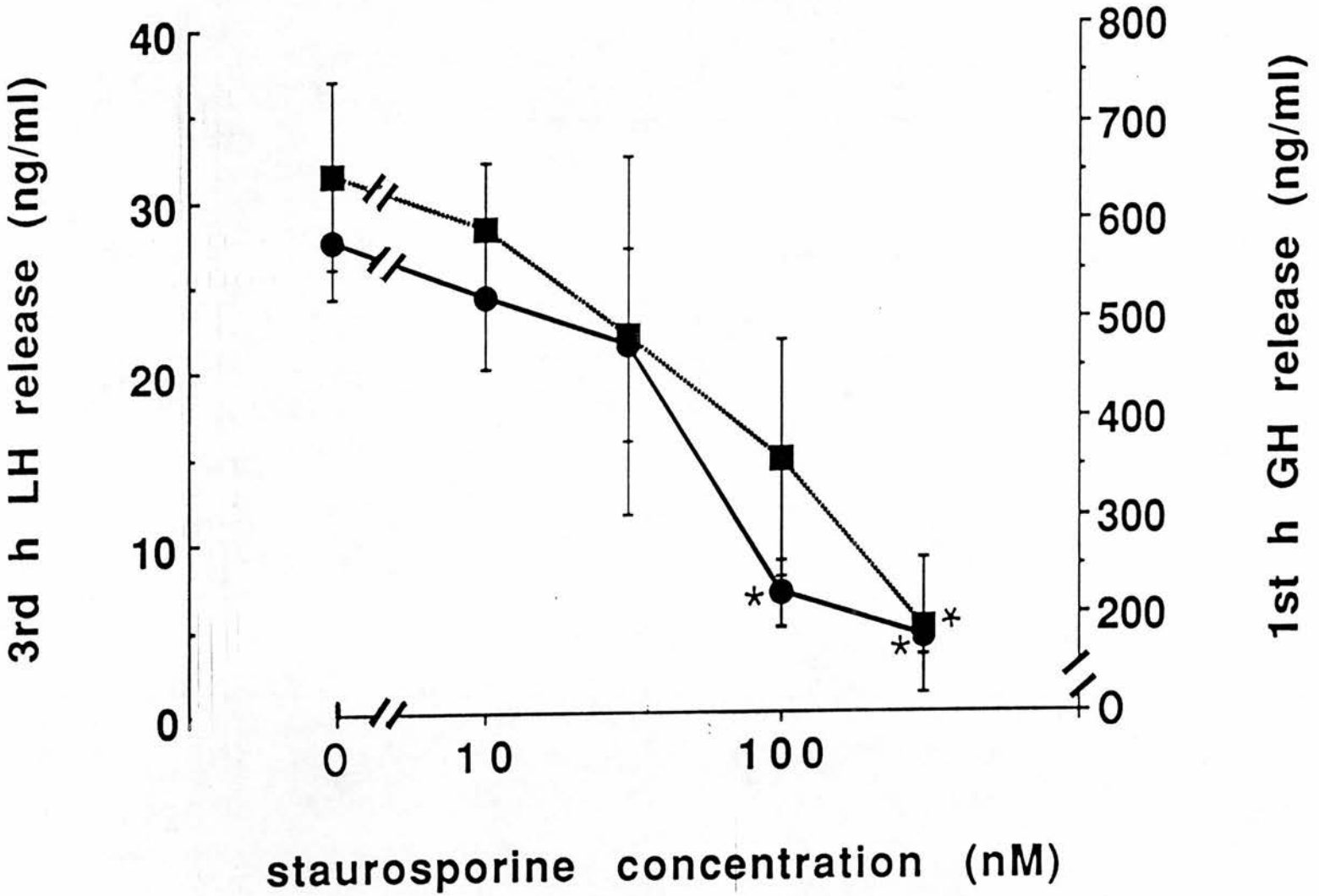

LH release (ng/ml)
GH release (ng/ml)
30
20
10
0
3000
2000
1000
0
*
basal
1
2
3
consecutive hourly incubations with PDBu

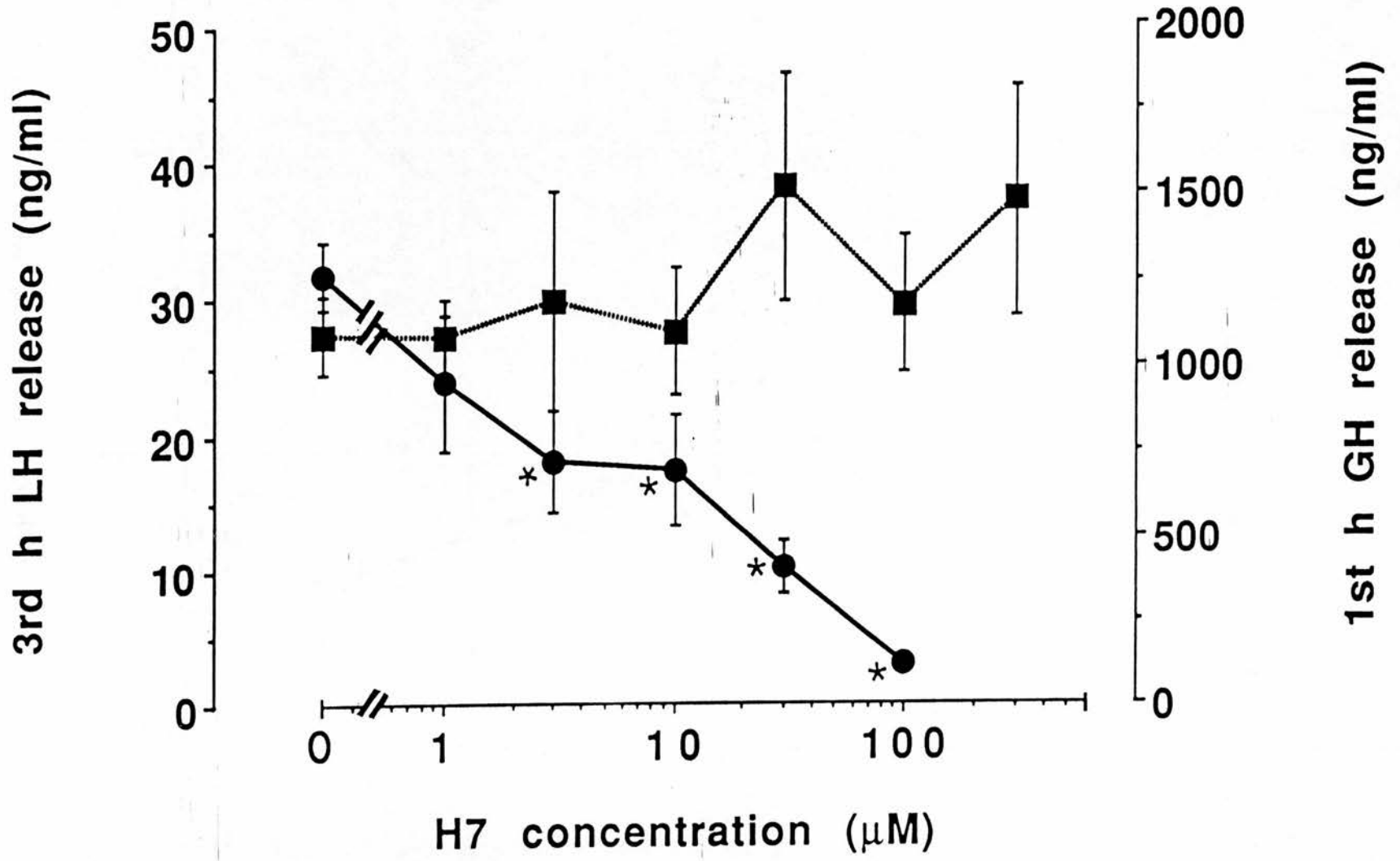

LH release (fold of basal h)
40
30
20
10
0
0
10
100
1000
10000
PKC activator concentration (nM)

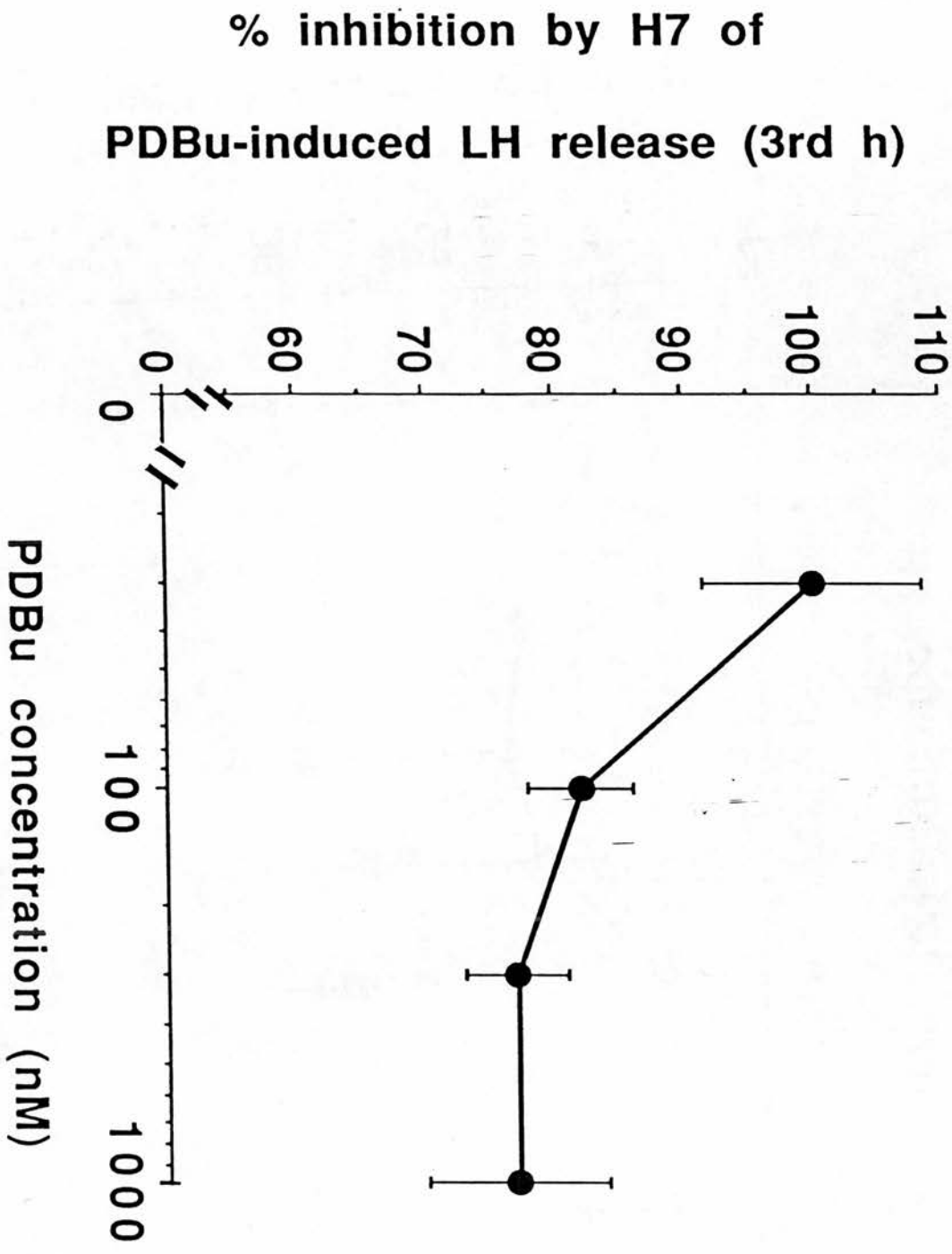

GH release (fold of basal h)
4
3
2
1
0
0
10
100
1000
10000
PKC activator concentration (nM)

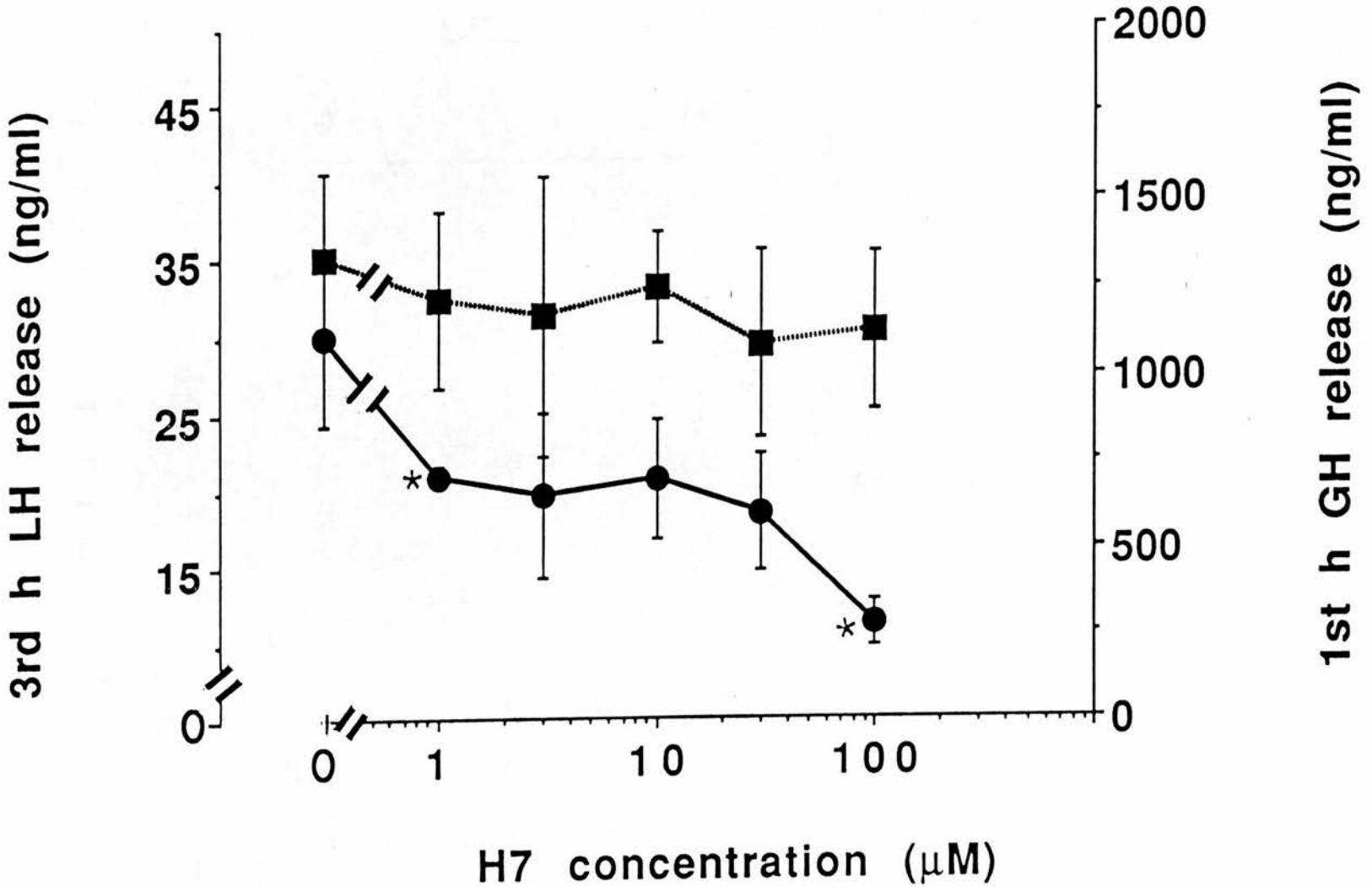

LH release (ng/ml)
9
8
7
6
5
4
0
*
*
1
2
3
consecutive hourly incubations with DOG


300
200
100
0
GH release (ng/ml)
1
2
3
consecutive hourly incubations with DOG


3rd h LH release (ng/ml)
40
30
20
10
0
800
700
600
500
400
300
200
0
1st h GH release (ng/ml)
0
10
100
staurosporine concentration (nM)
*
*
*


3rd h LH release (ng/ml)
50
40
30
20
10
0
2000
1500
1000
500
0
1st h GH release (ng/ml)
0
1
10
100
H7 concentration (μM)
*
*
*
*


% inhibition by H7 of
PDBu-induced LH release (3rd h)
0
60
70
80
90
100
110
0
100
1000
PDBu concentration (nM)


3rd h LH release (ng/ml)
45
35
25
15
0
2000
1500
1000
500
0
1st h GH release (ng/ml)
0
1
10
100
H7 concentration (μM)
*
*

Oestradiol-17ß modulates the actions of pharmacologically distinct forms of protein kinase C in rat anterior pituitary cells

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ABSTRACT

Phorbol ester-induced release of LH and GH from rat anterior pituitary tissue *in vitro* is differentially inhibited by some, but not other, inhibitors of protein kinase C (PKC), suggesting that pharmacologically distinct species of PKC may have different functional roles in these cells. Since stimulus-induced anterior pituitary hormone release can be enhanced by oestradiol-17ß (OE_2) pretreatment, we investigated the effect of OE_2 treatment of long-term (4 weeks) ovariectomized rats on the amount, activity and cellular actions of pharmacologically distinct PKC species in rat anterior pituitary tissue. Here we report that OE_2 treatment enhanced phorbol 12,13-dibutyrate (PDBu)-induced LH but not GH release measured *in vitro*. This effect of OE_2 on LH release may involve synthesis of additional PKCs that are not targeted by the synthetic diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DOG). Measurements of anterior pituitary PKC activity and [^{3}H]phorbol ester-binding studies suggested that the facilitatory action of OE_2 on LH release may occur, at least in part, by altering the quantity and activity of PKC(s). Our results also demonstrate that the OE_2-induced PKC(s) which facilitate LH release may be of the type that are not dependent upon raised intracellular Ca^{2+} for their activation and display distinct pharmacological properties (being readily activated by PDBu, but not by DOG, and are staurosporine-sensitive but H7-insensitive). This facilitatory action of OE_2 on PKC-induced LH release does not appear to involve OE_2-induced changes in the affinity of existing PKC(s) for PDBu, or changes in the amount of releasable LH in the pituitary prior to the stimulus.

INTRODUCTION

The release of a number of anterior pituitary hormones can be induced *in vitro* by activators of protein kinase C (PKC), such as phorbol esters (Smith & Vale, 1981; Johnson & Mitchell, 1989) and diacylglycerols (Conn, Ganong, Ebeling *et al.* 1985; Negro-Villar & Lapetina, 1985), suggesting that PKC activation may have a physiological role in triggering pituitary hormone release (McArdle, Huckle & Conn, 1987; Stojilkovic, Chang, Ngo & Catt, 1988). There is increasing evidence that different PKC-mediated responses (and perhaps certain isoforms of PKC) may be distinguished by their differential sensitivity towards various activators and inhibitors (Johnson & Mitchell, 1989; Nakadate, Yamamoto, Aizu *et al.* 1989; Mitchell, MacEwan, Johnson & Thomson, 1990; MacEwan & Mitchell, 1991; MacEwan, Mitchell, Johnson & Thomson, 1991; Ryves, Evans, Olivier *et al.* 1991; MacEwan, Simpson, Mitchell *et al.* 1992). We have shown, for example, that phorbol ester-induced luteinizing hormone (LH) but not growth hormone (GH) release from pro-oestrous rat anterior pituitary tissue can be readily inhibited by low concentrations of the PKC inhibitor, H7, despite the release of both hormones being similarly sensitive to other PKC inhibitors such as staurosporine (Johnson & Mitchell, 1989).

The responsiveness of gonadotrophs to secretagogues, such as luteinizing hormone-releasing hormone (LHRH) (Aiyer & Fink, 1974; Drouin, Legace & Labrie, 1976; Kamel, Balz, Kubajak & Schneider, 1987; Turgeon & Waring, 1981), depolarizing concentrations of potassium (Liu & Jackson, 1988) and activators of PKC (Liu & Jackson, 1988; Fahmy, Das & Bourne, 1989; Audy, Boucher & Bonnin, 1990; Drouva, Gorenne, Laplante *et al.* 1990) can be markedly altered by the prior presence of gonadal steroids, suggesting that steroids modulate several aspects of stimulus-secretion coupling in these cells. We therefore investigated the possibility that steroid modulation of pituitary responsiveness may involve an action on the activity of pharmacologically distinct forms of PKC that are involved in mediating hormone release.

MATERIALS AND METHODS

Animals

Adult female COB Wistar rats (approximately 200 g, Charles River U.K. Ltd, Margate, Kent, U.K.) were maintained under controlled lighting and temperature with free access to food pellets (CRM, Labsure, Manea, Cambs, U.K.) and tap water. Rats were bilaterally ovariectomized under halothane anaesthesia and left for a period of 4 weeks before being randomly divided into two experimental groups. The group treated with oestradiol-17ß (OE_2) received subcutaneous implants in the dorsal neck with 1 cm long silicone elastomer capsules (Dow-Corning Corporation, Midland, MI, USA), with an external diameter of 0.125 inches and internal diameter of 0.078 inches, containing 0.5 cm of crystallized oestradiol-17ß, and the control (i.e. OE_2 untreated) group received equivalent implants but with empty capsules (Dzuik & Cook, 1966; Henderson, Baker & Fink, 1977). Animals were killed in the morning of the 5th day after implantation.

Chemicals

Phorbol 12,13-dibutyrate (PDBu), 1,2-dioctanoyl-*sn*-glycerol (DOG) (Sigma Chemical Co Ltd, Poole, Dorset, U.K.), ionomycin and staurosporine (Novabiochem, Nottingham, Notts, U.K.) were made up as stock solutions in dimethylformamide (DMF). The maximum concentration of DMF (0.5%, v/v) was used in control experiments and had no effect. [20-^{3}H(N)]Phorbol 12,13-dibutyrate (specific activity = 19.1 Ci/mmol) and ATP-γ-^{35}S (specific activity = 1256 Ci/mmol) were purchased from Du Pont, Dreieich, Germany. 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H7; Gibco-BRL, Paisley, Strathclyde, U.K.), leupeptin (Boehringer Mannheim, Lewes, U.K.) and trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64; Sigma) were all made up as stock solutions in distilled H_2O. Oestradiol-17ß and phenylmethylsulphonyl fluoride (PMSF) were obtained from Sigma and phosphatidyl serine (sodium salt) from Lipid Products, Nutfield, Surrey, U.K.

Nonidet P-40 was from Novabiochem and Analar grade laboratory chemicals were obtained from BDH, Dagenham, Essex, U.K.

Hormone secretion experiments

The methods were based upon those used by Pickering and Fink (1976). Anterior pituitary glands were bisected and preincubated for 30 min (37°C, 95% O_2/5% CO_2; 1 hemipituitary/flask) in 2 ml pregassed and prewarmed Hepes-buffered minimal essential medium with Earle's salts (MEM; Gibco-BRL) after which the medium was discarded and replaced hourly with fresh MEM over a total period of 4 h. To examine the actions of secretagogues on hormone release, the tissue was incubated for an initial, basal hour in the presence of MEM only, followed by three consecutive hours (1st, 2nd and 3rd h) in the presence of a PKC activator (PDBu, DOG) or the calcium ionophore, ionomycin. In the experiments where the effects of kinase inhibitors (staurosporine, H7) on PKC-induced hormone release were examined, the inhibitor was present during the basal hour as well as the 1st, 2nd and 3rd hours, together with the activator. The medium was collected at the end of each hourly incubation and was stored at -20°C until it was radioimmunoassayed for LH and GH (Niswender, Midgley, Monroe & Reichert, 1968; Daane & Parlow, 1971). The standards used were NIDDK-rat LH-RP2 and NIDDK-rat-GH-RP-2.

Cytosolic PKC activity assay

Anterior pituitary PKC activity was determined as the phosphatidyl serine-dependent, histone III-S kinase activity induced by PKC activators using methods modified from those described by Wise, Glass, Chou *et al.* (1982) and Huang, Huang, Nakabayashi & Yoshida (1988). Anterior pituitary tissue was homogenized in 2 volumes of 20 mmol Tris-HCl/l (pH 7.5) containing 50 mmol 2-mercaptoethanol/l, 2 mmol EDTA/l, 1 mmol PMSF/l, 0.01% (w/v) leupeptin and 20 μmol E64/l. The homogenate was centrifuged (16,000 g, 20 min, 4°C) and the supernatant was collected and recentrifuged (16,000 g, 5 min, 4°C). The supernatant from the second spin was taken to represent cytosol and was partially purified by loading onto 0.5 ml

DE52 diethylaminoethyl cellulose (Whatman International Ltd, Maidstone, Kent, U.K.) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, CA, USA) at 4°C. The cytosol was then washed with six column volumes of homogenization buffer before eluting the partially purified PKC with three column volumes of buffer containing 150 mmol NaCl/l. Partially purified cytosolic PKC activity was measured in an assay mixture containing (final concentrations): 1.25 mmol $MgCl_2$/l, 100 mg phosphatidyl serine (sodium salt)/l, 0.04% (v/v) Nonidet P-40, 1.25 g histone III-S/l, 100 µmol ATP-γ-^{35}S/l (0.595 µCi/tube) and cytosol. Assay tubes also contained either 600 µmol $CaCl_2$/l (100 µmol free Ca^{2+}/l) or 5 mmol EGTA/l to give Ca^{2+}-dependent and Ca^{2+}-independent environments respectively. Phosphatidyl serine vesicles were prepared by drying the lipid from chloroform/methanol under a stream of N_2. The subsequent film of phosphatidyl serine was scraped into 20 mmol Tris-HCl/l (pH 7.5) + 0.5 mmol EGTA/l at a stock concentration of 400 mg/l, sonicated, then 0.16% (v/v) Nonidet P-40 was added. The mixture was vortexed before use. Protein kinase C activity was measured either in the absence of activator (i.e. basal activity) or in the presence of 1 µmol PDBu/l or 100 µmol DOG/l. All assay components and drugs were dissolved in 20 mmol Tris-HCl/l (pH 7.5) + 0.5 mmol EGTA/l except for DOG which was added to each assay tube in 1 µl DMF using a Hamilton microsyringe. Reactions were carried out at 30°C for 15 min and were stopped by quenching with 20 µl 0.1 mol ATP/l in 0.1 mol EDTA/l (pH 7.0). The quenched reaction mixture (50 µl) was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange paper (Whatman International Ltd) and washed (three x 10 ml, 2 min, room temperature) in 75 mmol H_3PO_4/l.

Kinase activity measured in the presence of phosphatidyl serine, 5 mmol EGTA/l (i.e. low free Ca^{2+}) and in the absence of PKC activator was taken to represent basal activity and was subtracted from all activator-stimulated levels. Calcium-independent kinase activity was taken to be the activity measured in the presence of 5 mmol EGTA/l and PKC activator (1 µmol PDBu/l or 100 µmol DOG/l).

The additional kinase activity measured with samples containing $CaCl_2$ + PKC activator was taken to represent calcium-dependent kinase activity.

Cytosolic [^{3}H]phorbol ester binding assay

The number of PKC molecules in OE_2-treated and untreated pituitary cytosol and their affinity for PDBu were assessed using a [^{3}H]PDBu-binding method described previously (Leach, James & Blumberg, 1983). Briefly, anterior pituitaries were homogenized in 2 volumes of 50 mmol Tris-HCl/l (pH 9.0) containing 1 mmol PMSF/l and 1 mmol $MnCl_2$/l then centrifuged (100,000 g, 1 h, 4°C). The supernatant was collected and recentrifuged (120,000 g, 1 h, 4°C) and the supernatant from the second spin was regarded as cytosol. Cytosol was diluted in assay buffer (50 mmol Tris-HCl/l (pH 7.4), 4 g essential fatty acid-free bovine serum albumin/l, 1 mmol $CaCl_2$/l and 75 mmol magnesium acetate/l) to a concentration which gave total binding of approximately 10% of total radioactivity present. The assay constituents were 1 g phosphatidyl serine (sodium salt)/l, [^{3}H]PDBu (2 - 500 nmol/l) and diluted cytosol. Phosphatidyl serine vesicles were prepared by dissolving the lipid in chloroform, drying under N_2, reconstituting into assay buffer, vortexing and sonicating. Total binding was measured in the presence of 0.5% DMF whilst non-specific binding was measured in the presence of 20 µmol PDBu/l. After a 30-min incubation (37°C), protein was precipitated by adding 100 µl 12 g bovine gamma-globulin/l and 300 µl 24% (w/v) polyethyleneglycol 8000 (4°C, 20 min). The precipitate was pelleted by centrifugation (12,000 g, 5 min, 4°C), the supernatant removed by aspiration and the radioactivity in each pellet counted.

Protein assay

Protein content in anterior pituitary cytosol, and in DEAE cellulose-purified cytosol was measured using a Pierce protein assay kit (Pierce, Chester, Cheshire, U.K.). This method used an assay reagent based on the Bradford method consisting of Coomassie blue G-250, phosphoric acid, methanol, water and solubilizing agents.

When Coomassie Blue binds to proteins in an acidic solution, an absorbance shift from 465 to 595 nm occurs (Bradford, 1976).

Data analysis

All values are expressed as mean ± S.E.M. Statistical analyses were carried out using the Mann-Whitney U-test or in the case of time-course data, by both 2-way and 1-way Analysis of Variance, followed by Duncan's New Multiple Range test on significantly different groups. The concentrations of the kinase inhibitors producing 50% inhibition of the stimulus-induced response (IC_{50} values) were determined by fitting the data with a non-linear, error-weighted, iterative curve-fitting programme, P.fit (Biosoft, Cambridge, Cambs., U.K.)

RESULTS

Effects of oestradiol-17ß on hormone release induced by PKC activators

Consecutive hourly incubations with PDBu (300 nmol/l) significantly increased net LH and GH release from anterior pituitary tissue taken from both OE_2-treated and untreated ovariectomized rats (Figs. 1 and 2). The baseline release of both hormones was determined in parallel control incubations for 4 h and the corresponding values were subtracted from all stimulated values to allow presentation of net stimulus-induced responses. In OE_2-treated but not untreated tissue there was a time-dependent increase in LH release in response to PDBu ($F(3,36) = 37.35$, $p \leq 0.0001$ and $F(3,36) = 1.73$, $p = 0.178$, respectively (Fig. 1a)). Statistically significant increments in LH release from OE_2-treated tissue occurred progressively at each of the hours of incubation with PDBu ($p < 0.05$). Thus, LH release from untreated tissue reached maximal levels by the 1st hour of PDBu incubation, whereas LH output from OE_2-treated tissue increased progressively with each successive PDBu incubation (in a manner similar to that seen with pro-oestrous tissue (Johnson, Mitchell & Fink, 1988)). The release of LH was significantly greater in OE_2-treated tissue than in ovariectomized controls in the 1st, 2nd and 3rd hours of PDBu stimulation ($p < 0.05$) but not in the basal hour (Fig. 1a).

The release of GH was increased by PDBu in both OE_2-treated and untreated tissue ($F(3,36) = 9.17$, $p \leq 0.0001$ and $F(3,36) = 5.47$, $p \leq 0.0034$ respectively) with statistically significant increments over basal ($p < 0.05$) in the 1st, 2nd and 3rd hours of PDBu in each group (Fig. 2a). There were however no significant differences in GH release between any of the hours of PDBu in either group or between OE_2-treated and untreated groups at any point (Fig. 2a). These data are consistent with OE_2 having a facilitatory action on the mechanism by which phorbol ester activators of PKC can induce LH but not GH release.

In a manner similar to PDBu, an alternative PKC activator DOG (200 µmol/l) also induced a significant increase in LH release from pituitary tissue from both untreated and OE_2-treated animals (Fig. 1b). Analysis of the raw data failed to show a statistically significant time-dependent increase in overall LH output from either OE_2-treated or untreated tissue in the presence of DOG. However, the corresponding basal LH release in each hour (assessed in parallel control incubations) showed a time-dependent decrease. When these values were subtracted to yield net DOG-induced LH release, non-parametric analysis (Mann-Whitney U-test) revealed a significant increment in LH release at each hour of incubation with DOG ($p < 0.05$). There were no significant differences between DOG-induced LH release from OE_2-treated or untreated tissue at any time point. Using pituitary tissue from untreated ovariectomized rats, the magnitude of DOG-induced LH release was not significantly different from PDBu-induced LH release at any hour of incubation with PKC activator. However, DOG was unable to mimic the enhanced effect of PDBu on LH release from OE_2-treated tissue as the magnitude of DOG-induced LH release from OE_2-treated and untreated tissue was similar.

In contrast to the effects of PDBu, DOG (at concentrations up to 200 µmol/l) was unable to induce GH release from either OE_2-treated or untreated tissue (Fig. 2b), suggesting that the PKC(s) that mediate GH and LH release differ in their susceptibility to activation by DOG.

In a further set of experiments, we examined the effects of the kinase inhibitors, staurosporine and H7 on PKC activator-induced hormone release from pituitary tissue from OE_2-treated and untreated ovariectomized rats (Table 1). The release of LH induced by PDBu (300 nmol/l) from both OE_2-treated and untreated ovariectomized rat pituitary tissue was significantly inhibited by 300 nmol staurosporine/l ($p < 0.05$). However, in contrast to previous results obtained with pro-oestrous pituitary tissue, where PDBu-induced LH release was readily inhibited by H7 (IC_{50} = 15.6 ± 4.5 µmol/l, n = 5) (Johnson & Mitchell, 1989), H7, at concentrations up to 30 µmol/l, was unable to significantly inhibit either DOG- or PDBu-induced LH release from anterior pituitary tissue taken from either OE_2-treated or untreated ovariectomized rats (Table 1). Phorbol-ester induced GH release was similarly inhibited by staurosporine, but was insensitive to H7 (up to 30 µmol/l). Neither staurosporine nor H7 at the concentrations used had any significant effect on basal LH or GH release (data not shown).

Effects of oestradiol-17ß on protein kinase C activity and [^{3}H]phorbol ester binding sites

We investigated the possibility that OE_2 treatment may enhance PKC-induced LH release by altering anterior pituitary PKC activity. Using histone III-S as a substrate, PDBu (1 µmol/l) was able to elicit approximately equal proportions of Ca^{2+}-dependent and -independent PKC activity in pituitary cytosol from untreated ovariectomized rats (Table 2). With OE_2 treatment there was approximately a 60% increase in both Ca^{2+}-dependent and -independent PDBu-induced PKC activity. In both treatment groups, DOG (100 µmol/l) apparently induced relatively less Ca^{2+}-dependent and -independent cytosolic kinase activity than PDBu (Table 2). However, direct comparisons of the kinase activity elicited by DOG and PDBu cannot be made unless comprehensive concentration response curves are available for each of these agents . Similar to the results seen using PDBu, DOG-stimulated Ca^{2+}-dependent PKC activity approximately doubled with OE_2 treatment. Whilst

there was also a slight increase in DOG-stimulated Ca^{2+}-independent PKC activity with OE_2 treatment, this was not statistically significant. It seems likely that such PDBu-sensitive, DOG-insensitive, Ca^{2+}-independent PKCs rather than the PDBu-sensitive, DOG-sensitive, Ca^{2+}-dependent PKCs are responsible for the OE_2-induced facilitation of PDBu-evoked LH release observed.

Studies were carried out to examine the actions of H7 and staurosporine on PDBu-stimulated PKC activity in anterior pituitary tissue cytosol (Fig. 3). In anterior pituitary cytosol from untreated ovariectomized animals, PDBu (1 μmol/l)-stimulated, Ca^{2+}-dependent PKC activity was much more sensitive to inhibition by H7 (IC_{50} = 26.8 ± 3.8 μmol/l) than Ca^{2+}-independent activity (IC_{50} = 103.7 ± 23.0 μmol/l) (mean ± S.E.M. values, n = 4). In OE_2-treated tissue, although PDBu-stimulated activity was greater in magnitude, Ca^{2+}-dependent, PDBu-stimulated kinase activity was also found to be more sensitive to H7 (IC_{50} = 32.1 ± 3.3 μmol/l) than Ca^{2+}-independent activity (IC_{50} = 112.5 ± 24.5 μmol/l) (mean ± S.E.M. values, n = 6). In contrast, the potency of staurosporine at inhibiting both Ca^{2+}-dependent and -independent PKC activity did not differ significantly between any of the conditions. In cytosol from untreated rats, the IC_{50} values for staurosporine on Ca^{2+}-dependent and Ca^{2+}-independent PKC activity were 139 ± 65 nmol/l and 132 ± 46 nmol/l respectively (means ± S.E.M., n = 5). Corresponding values from OE_2-treated rats were 100 ± 10 nmol/l and 137 ± 51 nmol/l respectively (means ± S.E.M., n = 5). Since PDBu-induced LH and GH release from OE_2-treated and untreated ovariectomized tissue are relatively insensitive to inhibition by H7 (with no apparent inhibition at concentrations of 30 μmol/l (Table 1)), the results from the PKC activity assays suggest that those PKC(s) which induce both LH and GH release may not be dependent upon raised intracellular Ca^{2+} for their activation by phorbol esters.

Saturation binding studies using [^{3}H]PDBu at eight different concentrations were carried out to assess the number of PKC molecules and their affinity for phorbol ester, with or without OE_2 treatment. Data were analysed by the non-linear, error-weighted curve-fitting programme P.fit (Biosoft). Treatment with OE_2

increased ($p < 0.05$, Mann-Whitney U-test) the number of [^{3}H]PDBu binding sites in anterior pituitary cytosol approximately 2-fold from 8.3 ± 0.6 pmol/mg protein to 18.7 ± 1.4 pmol/mg protein (means ± S.E.M., n = 5). The affinity (dissociation constant) of the binding sites for [^{3}H]PDBu was unaltered by OE_2 treatment, being 16 ± 3 nmol/l and 13 ± 1 nmol/l in untreated and OE_2-treated tissue respectively. It seems likely therefore, that the increase in PDBu-stimulated PKC activity observed after OE_2 treatment may occur as a result of an increase in pituitary PKC content, and not as a result of a change in the affinity of existing PKC(s) for PDBu.

Effects of oestradiol-17ß on ionomycin-releasable pools of LH and GH

It is possible that the facilitatory actions of OE_2 treatment on PKC-induced LH release may involve steroid-mediated enhancement of other steps in the process by which PKC can induce LH secretion, in addition to altering cytosolic PKC activity and content. To examine the possibility that OE_2 treatment can enhance LH release by increasing the amount of gonadotrophin available for release, we measured hormone release in response to maximally raised intracellular Ca^{2+} levels using the Ca^{2+} ionophore, ionomycin; a procedure which may be taken as an indirect measure of releasable pituitary hormone content. Over three consecutive hourly incubations, ionomycin (30 µmol/l) significantly increased LH and GH release from anterior pituitary tissue from both OE_2-treated and untreated ovariectomized animals (Fig. 4). In OE_2-treated and untreated tissue, ionomycin (30 µmol/l) caused significant release of LH ($F(3,12) = 27.66$, $p \leq 0.001$ and $F(3,12) = 12.59$, $p \leq 0.005$ respectively) (Fig. 4a). Compared with the pre-drug basal values, all individual time points (except for the third hour of ionomycin in OE_2-treated tissue) reached statistical significance ($p < 0.05$). When net ionomycin-induced increments in LH output were calculated by correcting for declining baseline, all increments were significant ($p < 0.05$, Mann-Whitney U-test). Treatment with OE_2 did not significantly affect responses to ionomycin at any time point (Fig. 4a). This evidence that OE_2 treatment enhances PDBu but not ionomycin-induced LH release, suggests

that OE_2 does not enhance PKC-induced LH release by increasing the size of the releasable LH pool.

Ionomycin also caused significantly increased release of GH in both OE_2-treated and untreated tissue ($F(3,12) = 48.27$, $p \leq 0.0001$ and $F(3,12) = 10.35$, $p \leq 0.0012$ respectively) (Fig. 4b). All time points with ionomycin (either with or without OE_2 treatment) showed significantly greater GH release than pre-drug basal ($p < 0.05$). There was no significant influence of OE_2 treatment on ionomycin-induced GH release at any time point, suggesting that OE_2 does not alter the readily releasable pool of GH.

Discussion

Eight isoforms of PKC (α, $ß_I$, $ß_{II}$, γ, δ, ε, ζ and η) have been sequenced; each having distinct tissue distributions (Nishizuka, 1988; Osada, Mizuno, Saido *et al.* 1990), indicating that the expression of various PKC isoforms is differentially regulated in different tissues. Anterior pituitary tissue is known to express α and ß but not γ isoforms (Naor, Dan-Cohen, Hermon & Limor, 1989; Naor, 1990) and evidence from Northern blots using oligonucleotide probes directed against unique sequence regions of the other PKC isoforms suggests that ε and ζ, but little δ isoform may also be present (E. Lutz, A. Ison, R. Mitchell & F.J. Thomson, unpublished observations). The results presented here suggest that OE_2 is an important regulator of PKC isoform expression in gonadotrophs, but not somatotrophs, with respect to the actions of PKC on hormone release *in vitro*. The increase in the amount of phorbol ester-stimulated pituitary cytosolic PKC activity with OE_2 treatment (Table 2) supports previous data (Drouva *et al.* 1990; Joubert-Bression, Brandi, Birman & Peillon, 1990) and suggests that the facilitatory action of OE_2 on phorbol ester-induced LH release involves an increase in the availability of PDBu-sensitive PKC molecules in anterior pituitary cells, including gonadotrophs. The increase in pituitary PKC content revealed by our [^{3}H]PDBu-binding studies, together with evidence that the facilitatory actions of OE_2 on stimulus-induced LH release can be

prevented by protein synthesis inhibitors (Debeljuk, Khar & Jutisz, 1978; Fahmy *et al.* 1989), suggests that OE_2 enhances PKC-stimulated LH release by inducing synthesis of additional PKC molecules. However, here we provide evidence that these additional OE_2-induced PKC(s) are pharmacologically distinct from those that mediated LH release prior to OE_2 treatment (Fig. 1 and Table 1). That is, in untreated tissue from ovariectomized rats, those PKCs that can induce LH release can be activated by both PDBu and DOG, whereas with OE_2 treatment there is an additional involvement of PDBu-sensitive but DOG-insensitive kinases. PKCs that are similarly resistant to activation by DOG are also involved in PDBu-induced GH release (Fig. 2). Cellular actions of PKC, elicited by phorbol esters, but not by DOG, have been described previously (Lacerda, Rampe & Brown, 1988; MacEwan & Mitchell, 1991; MacEwan, Johnson, Mitchell *et al.* 1992) and reduced potency of DOG as an activator of α-PKC has been reported (Sekiguchi, Tsukada, Ase *et al.* 1988) and is most marked under conditions of basal cytosolic Ca^{2+} (≤ 100 nmol/l) (MacEwan, Johnson, Mitchell *et al.* 1992).

The PKCs involved in both LH and GH release in the present experiments are also distinctive in their resistance to the PKC inhibitor H7, but not staurosporine (Table 1). Correspondingly, a component of PKC activity (the Ca^{2+}-independent fraction) from the anterior pituitary cytosol of both untreated and OE_2-treated ovariectomized rats was relatively resistant to H7. In contrast, PDBu-elicited, Ca^{2+}-dependent and -independent PKC activities measured in cytosol from ovariectomized rat hypothalamus and hippocampus displayed the expected sensitivities to H7. The IC_{50} values for the inhibition of Ca^{2+}-dependent and -independent activities by H7 in hypothalamus cytosol were 17.6 ± 8.1 µmol/l and 13.6 ± 4.4 µmol/l, respectively (means ± S.E.M., n = 4). Corresponding IC_{50} values for H7 measured in hippocampus cytosol were 19.6 ± 4.4 µmol/l and 21.3 ± 1.4 µmol/l (means ± S.E.M., n = 4). Oestradiol-17ß (OE_2) treatment had no significant effect upon the H7 sensitivity of PDBu-induced PKC activity in the hippocampus or hypothalamus. Interestingly, the priming effect of LHRH (Aiyer, Chiappa & Fink,

1974) is mediated by PKC(s) with the same unusual profile of sensitivity to PKC inhibitors (Johnson, Mitchell & Thomson, 1992). Phorbol 12,13-dibutyrate (PDBu)-induced GH release from intact pro-oestrous rat anterior pituitary tissue also shows H7 resistance (Johnson & Mitchell, 1989) whereas, in the same experiments (Johnson *et al.* 1988; Johnson & Mitchell, 1989), LH release was completely inhibited by H7 at a concentration of 30 μmol/l (as was used here). Thus, it appears that more than one pharmacologically distinct type of PKC(s) can mediate LH release under different circumstances and the involvement of particular PKC forms in this response may be dependent upon the gonadal steroid status of the animal. Progesterone can further enhance the actions of OE_2 on LHRH-induced gonadotrophin release (Mann & Barraclough, 1973 ; Turgeon & Waring, 1990) suggesting that gonadal steroids other than OE_2 are also required to regulate gonadotroph responsiveness. Thus, factors other than OE_2 may be required to influence the activity of the H7-sensitive PKCs that can induce LH release in the intact rat.

Some studies have suggested that gonadectomy or steroid treatment can alter GH release patterns measured in the rat *in vivo* (Jansson, Ekberg, Isaksson & Eden, 1984). However, in our model, OE_2 does not regulate either PKC actions on GH secretion or the size of the releasable pool of GH. Furthermore, there appears to be no clear relationship between GH secretory patterns in intact female rats and stage of the oestrous cycle (Clark, Carlsson & Robinson, 1987), consistent with our evidence that OE_2 does not play an important role in modulating anterior pituitary gland responsiveness with respect to PKC-mediated GH release. Oestradiol-17ß (OE_2) treatment of cultured pituitary cells *in vitro* has correspondingly been shown to enhance phorbol ester-induced LH but not GH release (Drouva *et al.* 1990).

Oestradiol-17ß (OE_2) has modulatory actions on several factors that may be involved in cellular signalling mechanisms, for example voltage-sensitive Ca^{2+} channels (Drouva, Rerat, Bihoreau *et al.* 1988) and LHRH receptor number (Gregg & Nett, 1989). Therefore, it is likely that OE_2 modulation of cellular signalling within

gonadotrophs may also occur at sites other than PKC and thereby contribute to the facilitatory actions of OE_2 on gonadotrophin release in intact animals. It is possible that such factors may contribute to the changes in the time-course of PDBu-induced LH release observed after OE_2 treatment. In some *in vitro* models, OE_2 treatment has positive effects on LH-ß gene transcription (Shupnik, Gharib & Chin, 1989), but *in vivo* replacement of OE_2 in long-term ovariectomized animals (as here) seems instead to reduce levels of mRNA for LH-ß (Counis, Corbani & Jutisz, 1983; Gharib, Bowers, Need & Chin, 1988; Papavisiliou, Zmeili, Herbon *et al.* 1986). Oestradiol-17ß (OE_2) can enhance LH synthesis and glycosylation and facilitate responses to LHRH (Liu & Jackson, 1977, 1990; Tang, 1980; Ramey, Highsmith, Wilfinger & Baldwin, 1987). However, the experiments here with ionomycin (Fig. 4) indicated that the facilitatory actions of OE_2 on phorbol ester-induced LH secretion do not appear to result from an OE_2-induced increase in releasable gonadotrophin content prior to a stimulus.

The identity of the PKC isoforms involved in LH and GH release is not yet known. However, since the known PKC isoforms differ in their dependency upon raised intracellular Ca^{2+} for activation they have been subclassified into two groups (Parker, Kour, Marais *et al.* 1989). The α, ß, and γ PKC forms (the A series) have been shown to be dependent upon raised intracellular Ca^{2+} for their activation, whereas activation of the δ, ε, ζ and η forms (the B series) appears to be independent of raised Ca^{2+}. Although the Ca^{2+}-dependent α- and ß-PKC isoforms have been implicated as having a role in gonadotrophin release (Naor *et al.* 1989), the H7-resistant PKCs which mediate both LH and GH release from ovariectomized rat tissue are readily activated by PDBu alone and do not overtly require elevated Ca^{2+} levels for activation. Since both Ca^{2+}-dependent and -independent PKC activities were increased by OE_2 treatment, it seems that other PKCs may be induced by OE_2, in addition to the PDBu-sensitive, DOG-insensitive, H7-resistant PKC relevant here in the increased responsiveness of gonadotrophs. Although the H7-resistant PKC would initially appear to be a B series (or related) isoform, there is evidence that the

degree of Ca^{2+} dependency exhibited by the A series isoforms may differ in the presence of certain fatty acids (Naor, Shearman, Kishimoto & Nishizuka, 1988; Shinomura, Asaoka, Oka *et al.* 1991). Furthermore, it has been suggested that the phosphorylation state of the ß-isoform can alter its Ca^{2+} dependency (Pelech, Samieim, Charest *et al.* 1991), so this classification of the known PKC isoforms according to their Ca^{2+} dependency may be less clear-cut than when originally conceived. Data obtained using highly purified or expressed recombinant preparations have shown that the δ- and ε-isoforms of PKC are more sensitive to H7 (Schaap & Parker, 1990; Uchida, Hagiwara & Hidaka, 1991) than those forms that induce LH and GH release in our model, suggesting that neither δ- nor ε-PKC is significantly involved in mediating LH or GH release in ovariectomized or OE_2-treated rat pituitary tissue. Furthermore, the ζ isoform is reported to be unresponsive to phorbol esters (Ono, Fujii, Ogita, Kikkawa *et al.* 1989), suggesting that ζ would not contribute to the present results. It seems therefore that the PKC isoform(s) which mediates PDBu-induced LH release from ovariectomized rat anterior pituitary tissue (and which is induced by OE_2 treatment) may be a covalently modified form of a PKC, a poorly characterized Ca^{2+}-independent isoform, such as η or an isoform of PKC (or closely related protein kinase) which has not yet been sequenced.

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Table 1 Effect of protein kinase C (PKC) inhibitors on PKC activator-induced LH and GH release from anterior pituitary tissue taken from ovariectomized rats with or without oestradiol-17ß (OE_2) treatment. All values are mean ± S.E.M. (number of determinations in parentheses).

	Untreated ovariectomized	**OE_2-treated**
	LH release (µg/l)	
PDBu	30.9 ± 4.1 (10)	308.0 ± 35.1 (6)
PDBu + staurosporine	8.0 ± 4.6 (8)*	94.6 ± 18.4 (8)*
PDBu + H7	29.8 ± 4.8 (6)	328.5 ± 25.2 (6)
DOG	22.0 ± 2.9 (5)	24.9 ± 2.8 (8)
DOG + staurosporine	8.9 ± 3.0 (4)*	9.9 ± 4.4 (4)*
DOG + H7	24.7 ± 2.9 (6)	21.9 ± 7.6 (6)
	GH release (µg/l)	
PDBu	1828.5 ± 256.6 (6)	1871.7 ± 290.1 (10)
PDBu + staurosporine	138.5 ± 56.9 (6)*	240.8 ± 184.6 (5)*
PDBu + H7	2079.2 ± 374.5 (6)	1866.8 ± 312.2 (5)

Hemipituitaries were incubated *in vitro* for 4 consecutive hours. In the basal hour there was either medium only or staurosporine (300 nmol/l) or H7 (30 µmol/l). In the 1st, 2nd, 3rd hour there was, in addition, either phorbol 12,13-dibutyrate (PDBu) (300 nmol/l) or dioctanoyl-*sn*-glycerol (DOG) (200 µmol/l). The data shows net LH release (i.e. with baseline subtracted) measured in the 3rd hour with PKC activator and net GH release measured in the 1st hour with PKC activator. *$p < 0.05$ compared with corresponding release from untreated tissue.

Table 2 Phorbol 12,13-dibutyrate- (PDBu) and 1,2-dioctanoyl-*sn*-glycerol (DOG) -stimulated protein kinase C (PKC) activity in anterior pituitary cytosol from ovariectomized rats with or without oestradiol-17ß (OE_2) treatment. All data are shown as mean ± S.E.M. with the number of determinations in parentheses.

Activator-induced PKC activity (pmol thiophosphate/mg protein per 15 min)	**Ovariectomized**	**Ovariectomized + OE_2-treated**
PDBu Ca^{2+}-independent	3056 ± 551 (4)	4829 ± 420 (7)*
PDBu Ca^{2+}-dependent	2806 ± 651 (4)	4535 ± 714 (7)*
DOG Ca^{2+}-independent	95 ± 36 (4)	138 ± 18 (5)
DOG Ca^{2+}-dependent	398 ± 26 (4)	807 ± 17 (5)*

These data show the PKC-activator-evoked phosphatidylserine-dependent, histone III-S kinase activity, measured in the presence of either phorbol 12,13-dibutyrate- (PDBu) (1 µmol/l) or 1,2-dioctanoyl-*sn*-glycerol (DOG) (100 µmol/l) at either < 3 nmol free Ca^{2+}/l or 100 nmol free Ca^{2+}/l. *$p < 0.05$ compared with corresponding activity measured from untreated tissue (Mann-Whitney U-test).

Figure 1

Effect of (a) phorbol 12,13-dibutyrate (PDBu) and (b) 1,2-dioctanoyl-*sn*-glycerol (DOG) on LH release from anterior pituitary tissue taken from oestradiol-17ß (OE_2) -treated (hatched bars) and untreated (open bars) ovariectomized rats. Tissue was incubated with medium only in the basal hour followed by three consecutive hourly incubations (1st, 2nd, 3rd h) in the presence of either (a) 300 nmol PDBu/l or (b) 200 µmol/DOG/l. Values are means ± S.E.M. (n = 4 - 10). The data represent net hormone release, i.e. baseline release (determined in parallel control incubations over 4 hours) was subtracted. Initial baseline secretion of LH was in the range of 12 - 35 mg/l and was not different between tissue from OE_2-treated and untreated ovariectomized rats. LH release from both OE_2-treated and -untreated tissue was significantly increased above baseline levels during all hours of incubation with either PDBu or DOG (*$p < 0.05$, Duncan's test or Mann-Whitney U-test). Phorbol 12,13-dibutyrate (PDBu)-, but not DOG-induced LH release was significantly enhanced by OE_2 treatment (+$p < 0.05$ compared with LH release from untreated tissue at each hour of stimulation with that PKC activator; Duncan's test or Mann-Whitney U-test)

Figure 2

Effect of (a) phorbol 12,13-dibutyrate (PDBu) and (b) 1,2-dioctanoyl-*sn*-glycerol (DOG) on GH release from anterior pituitary tissue taken from oestradiol-17ß (OE_2) -treated (hatched bars) and -untreated (open bars) ovariectomized rats. Tissue was incubated with medium only in the basal hour followed by three consecutive hourly incubations (1st, 2nd, 3rd h) in the presence of either 300 nmol PDBu/l or 200 µmol DOG/l. Values are means ± S.E.M. (n = 4 - 10). The data represent net hormone release, i.e. baseline release (determined in parallel control incubations over 4 hours) was subtracted. Growth hormone release was increased in the presence of PDBu, but not DOG during all hours of incubation with PKC activator (*$p < 0.05$,

Duncan's test or Mann-Whitney U-test). There was no significant influence of OE_2 treatment on GH release in the presence of PDBu or DOG (Duncan's test or Mann-Whitney U-test).

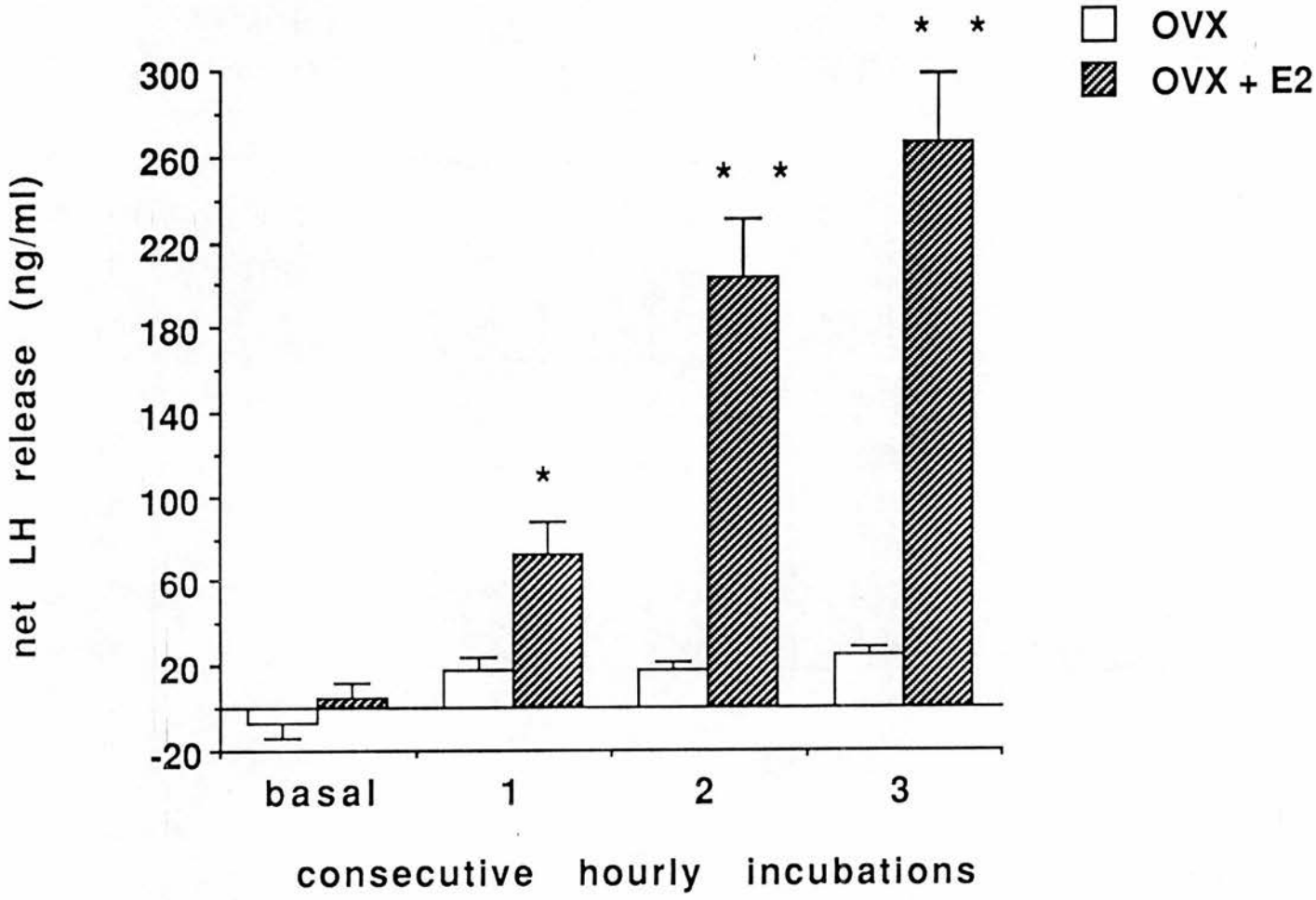
Figure 3

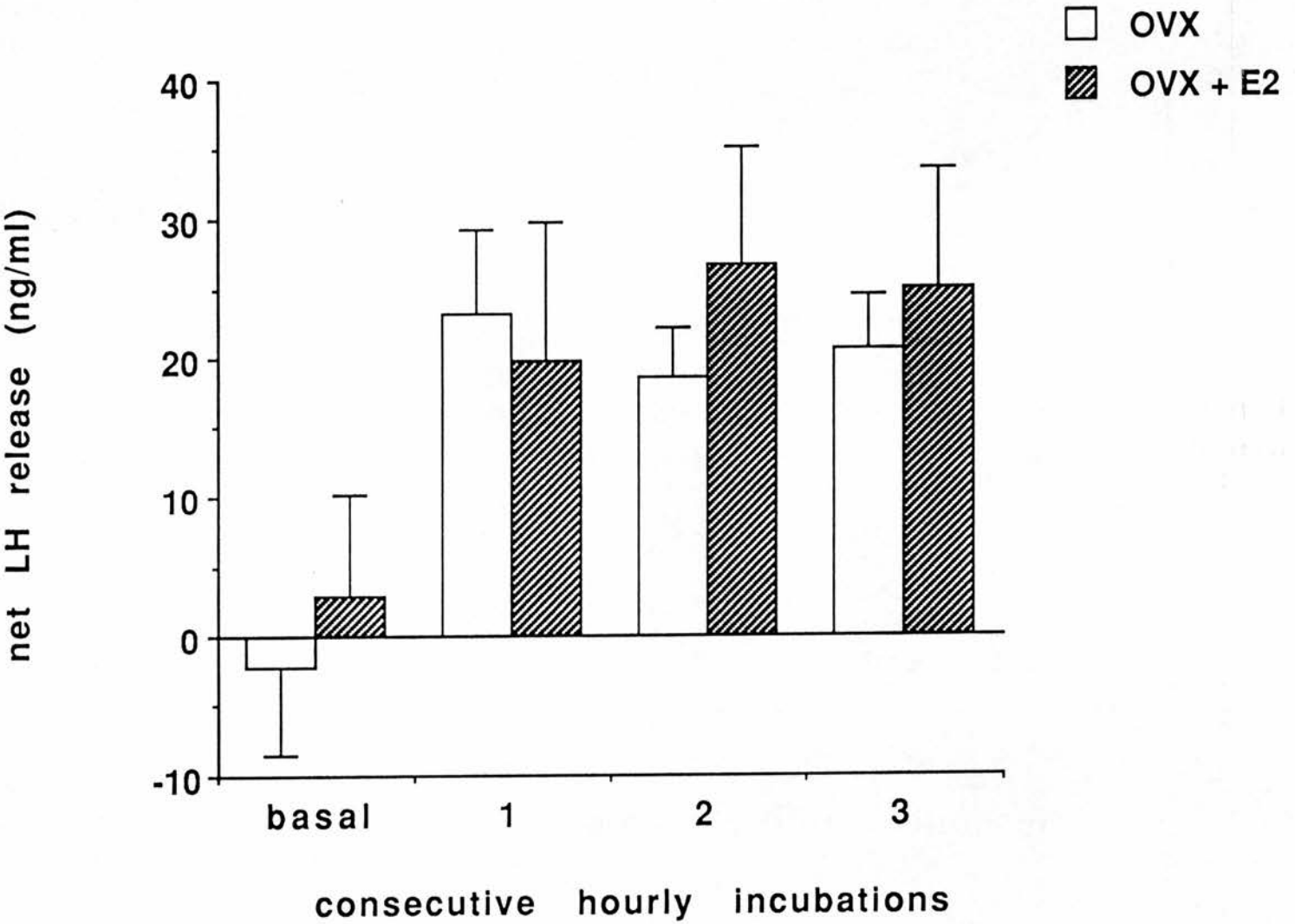
Effect of the protein kinase C (PKC) inhibitor H7 on phorbol 12,13-dibutyrate (PDBu) -stimulated Ca^{2+}-independent (●/○) and Ca^{2+}-dependent (■/❑) cytosolic PKC activity from ovariectomized rat anterior pituitary tissue with (closed symbols) or without (open symbols) oestradiol-17ß (OE_2) treatment. Cytosolic PKC activity was partially purified and the phosphatidylserine-dependent, histone III-S kinase activity measured with 1 µmol PDBu/l, and various concentrations of H7, in the presence (100 µmol/l) or absence (< 3 nmol/l) Ca^{2+} as described in the methods section. Each point on the curves is the mean ± S.E.M. of 4 - 6 determinations. The IC_{50} values (concentration of the inhibitor causing 50% inhibition of the activator-induced response) were obtained by using a non-linear, error-weighted curve fitting programme (P.fit, Biosoft) and are expressed as the mean ± S.E.M. (with the number of determinations in parentheses). Calcium-independent PDBu-induced PKC activity in ovariectomized rat anterior pituitary cytosol was less sensitive to inhibition by H7 than Ca^{2+}-dependent activity, with IC_{50} values of 104 ± 23 µmol/l and 27 ± 4 µmol/l respectively (n = 4). This profile of H7-sensitivity was not significantly altered by OE_2 treatment, where the corresponding IC_{50} values were 113 ± 25 µmol/l and 32 ± 2 µmol/l respectively (n = 6).

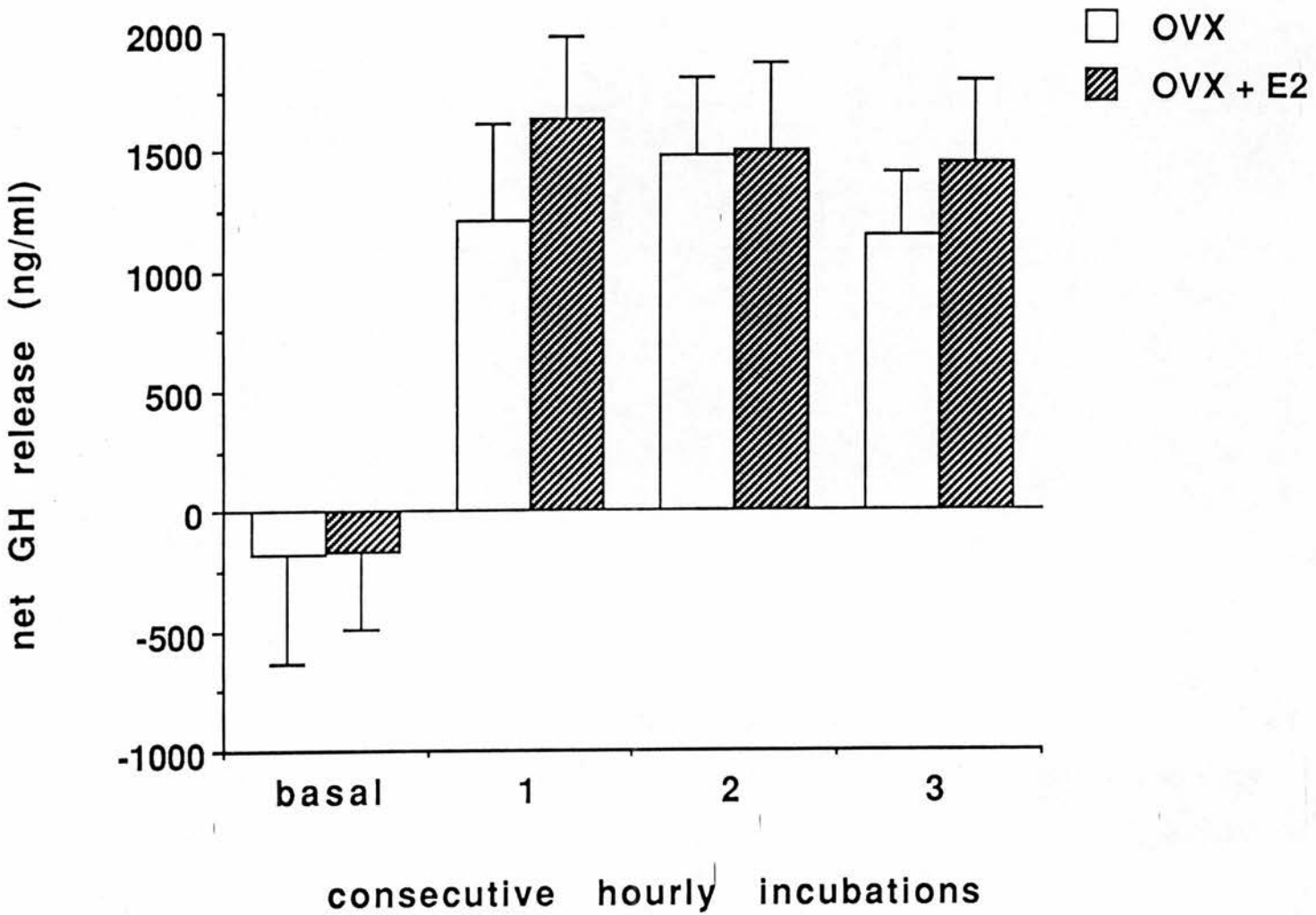
Figure 4

Effect of ionomycin on (a) LH and (b) GH release from anterior pituitary tissue taken from oestradiol-17ß (OE_2) -treated (hatched bars) and -untreated (open bars) ovariectomized rats. Tissue was incubated with medium only in the basal hour followed by three consecutive hours (1st, 2nd, 3rd h) in the presence of 30 µmol ionomycin/l. Values are means ± S.E.M. (n = 4 - 6). The data represent net

hormone release, i.e. baseline release (determined in parallel control incubations over 4 hours) was subtracted. Both LH and GH release were significantly increased during all hours of incubation with ionomycin (*$p < 0.05$, Duncan's test or Mann-Whitney U-test). Release of both LH and GH in the presence of ionomycin was not significantly influenced by OE_2 treatment.


net LH release (ng/ml)
300
260
220
180
140
100
60
20
-20
basal
1
2
3
consecutive hourly incubations
OVX
OVX + E2
*
*
*
*
*
*


OVX
OVX + E2
net LH release (ng/ml)
40
30
20
10
0
-10
basal
1
2
3
consecutive hourly incubations


OVX
OVX + E2
net GH release (ng/ml)
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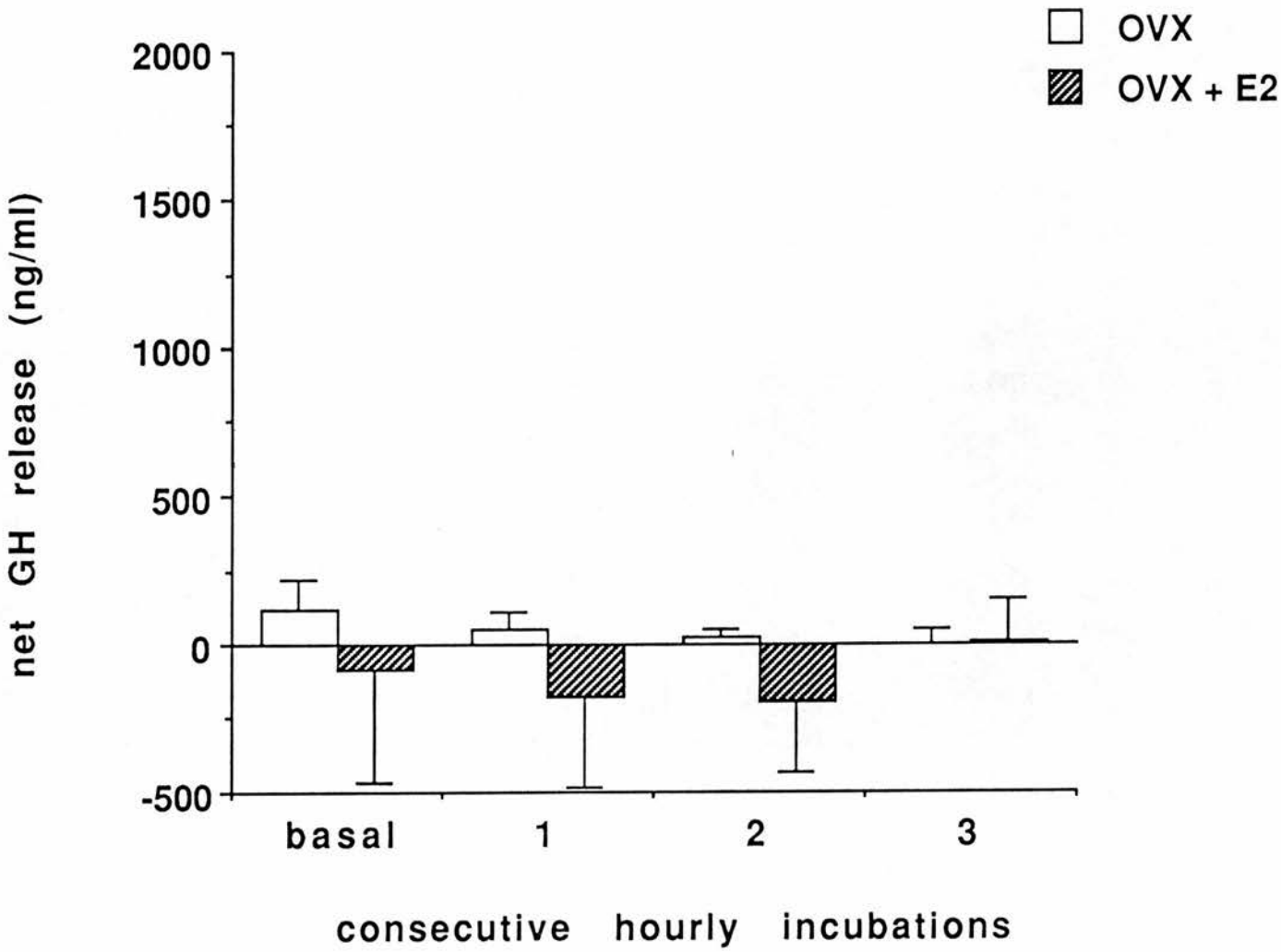
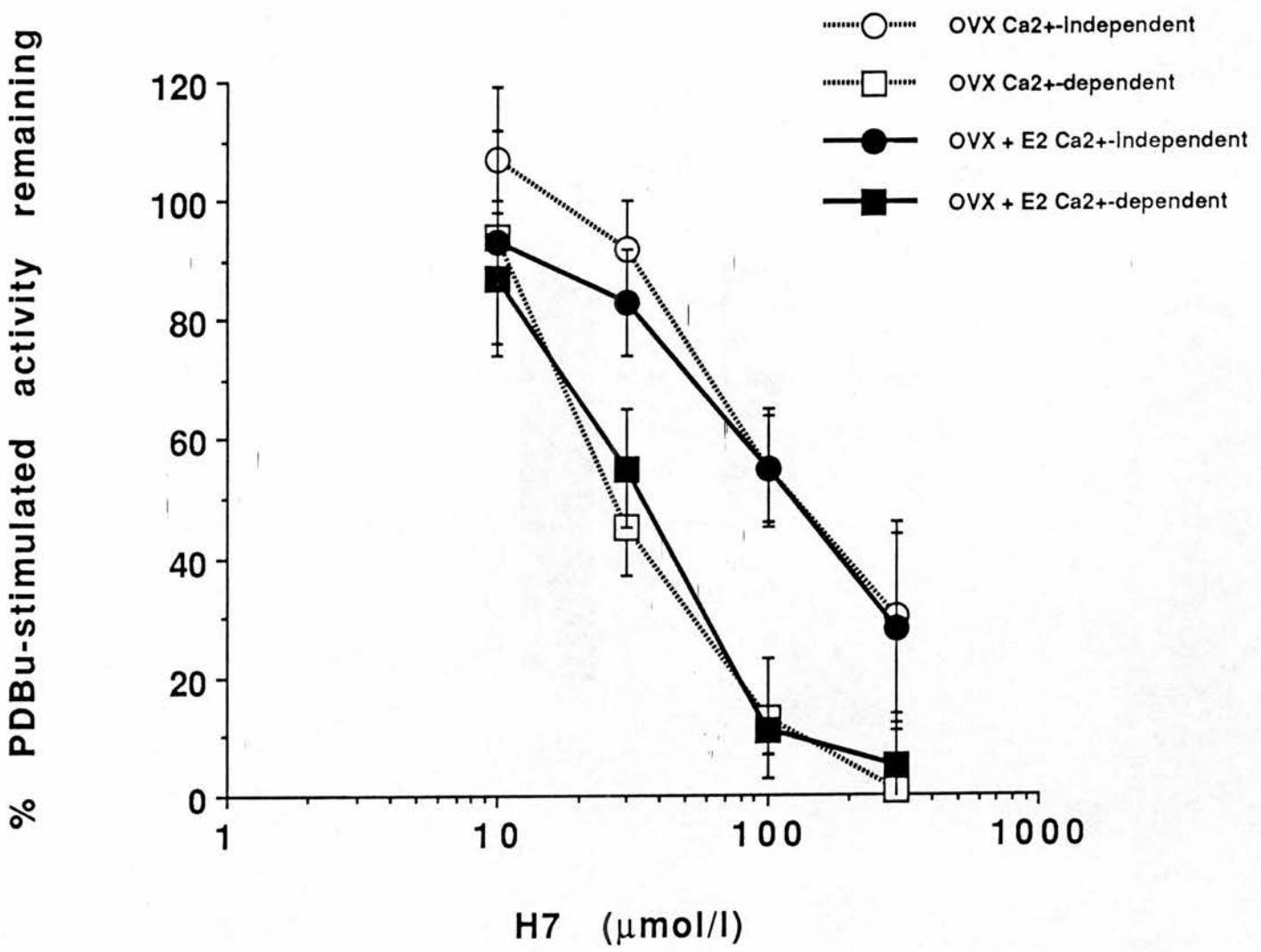
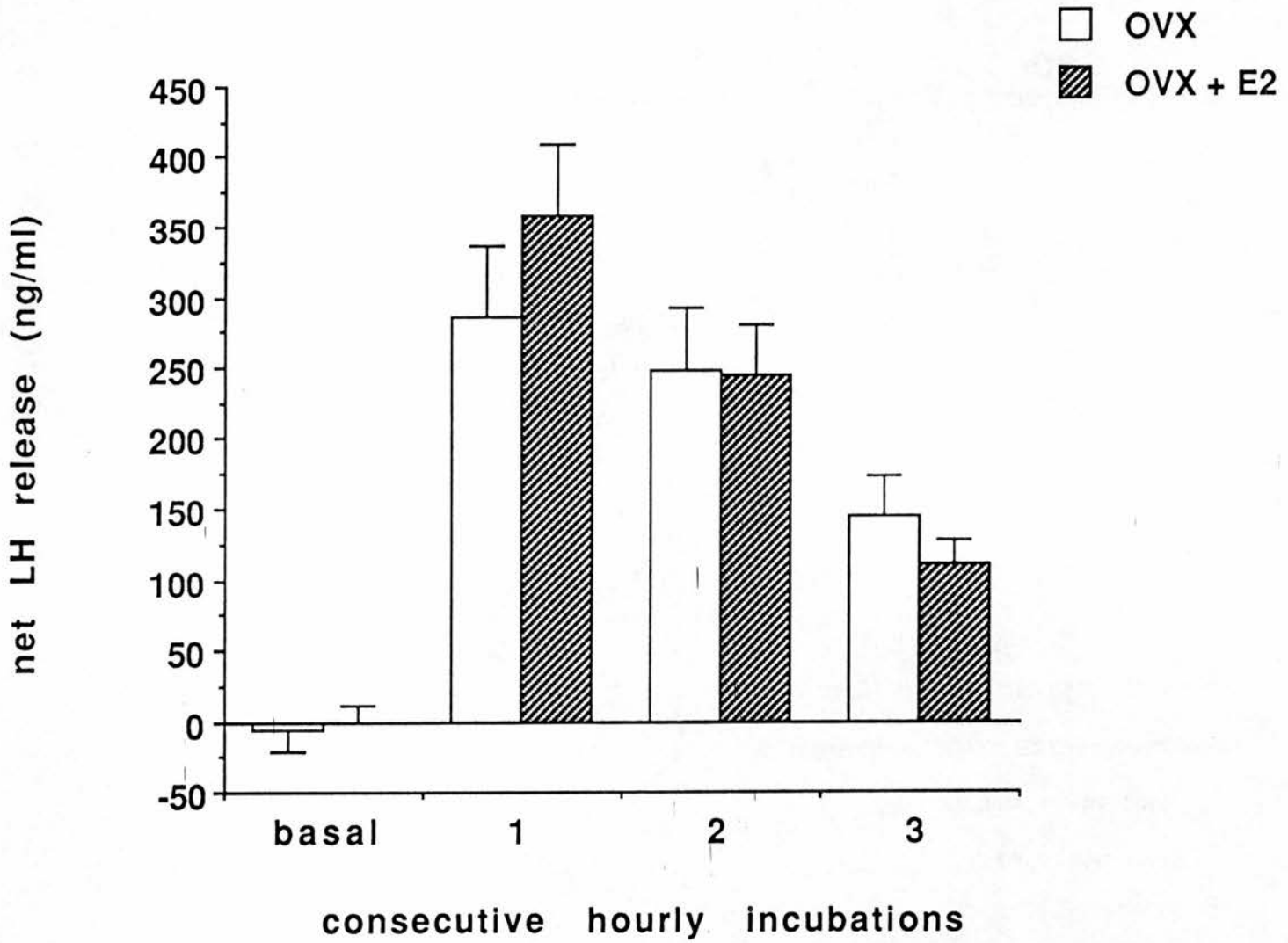
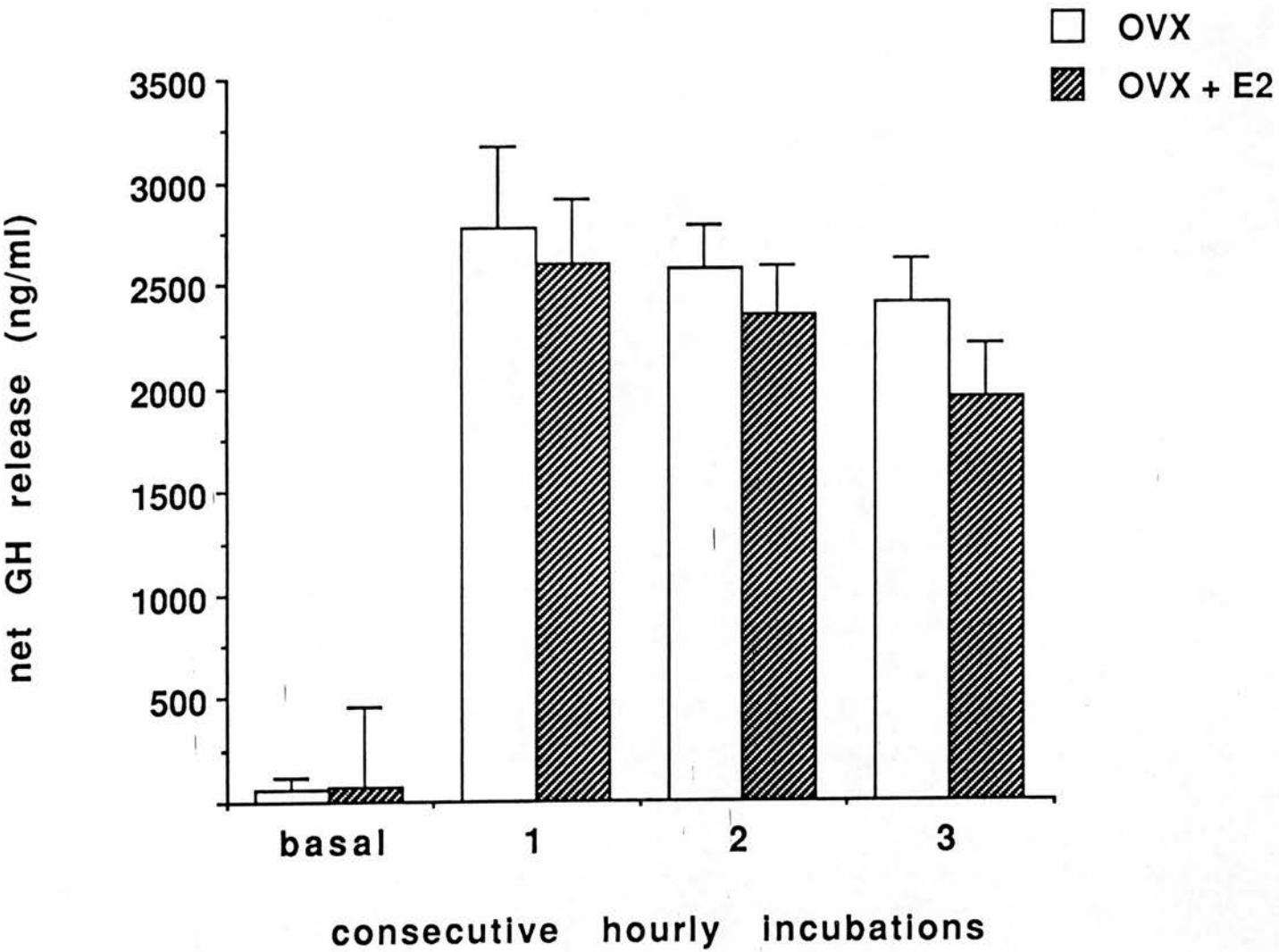
1500
1000
500
0
-500
-1000
basal
1
2
3
consecutive hourly incubations


OVX
OVX + E2
2000
1500
1000
500
0
-500
net GH release (ng/ml)
basal
1
2
3
consecutive hourly incubations


% PDBu-stimulated activity remaining
120
100
80
60
40
20
0
1
10
100
1000
H7 (µmol/l)
OVX Ca2+-Independent
OVX Ca2+-dependent
OVX + E2 Ca2+-Independent
OVX + E2 Ca2+-dependent


OVX
OVX + E2
net LH release (ng/ml)
450
400
350
300
250
200
150
100
50
0
-50
basal
1
2
3
consecutive hourly incubations


OVX
OVX + E2
net GH release (ng/ml)
3500
3000
2500
2000
1500
1000
500
basal
1
2
3
consecutive hourly incubations

P.C. 7

A possible role for phospholipase A_2 in phorbol ester-induced release of hormones from rat anterior pituitary tissue *in vitro*

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Stimulation of luteinizing hormone (LH) and growth hormone (GH) release by phorbol 12,13-dibutyrate (PDBu) *in vitro* (Johnson & Mitchell, 1989) is consistent with a physiological role for protein kinase C (PKC) in triggering or modulating release of these hormones. Differences in the pharmacology and time course of PDBu-induced LH and GH release suggest that PKC(s) may influence different targets in gonadotrophes and somatotrophes. Since arachidonic acid (AA) has been reported to induce LH release (Naor *et al.* 1981), we investigated the possibility that AA production by phospholipase A_2 (PLA_2) may have a role in PKC-mediated LH and GH release.

Female COB/Wistar rats that had been maintained under controlled lighting and temperature were killed at 13.00 h on the appropriate day of the oestrous cycle. Anterior pituitary glands were removed and hemisected. The release of LH and GH *in vitro* was measured as described previously (Johnson & Mitchell, 1989). Phospholipase A_2 activity was determined in anterior pituitary tissue preincubated for 1 h with [^{3}H]arachidonic acid in minimal essential medium at 37 °C under 95% O_2/5% CO_2. After extensive washing, stimuli were applied for 1 h and the labelled lipids and metabolites separated on octadecylsilyl silica (Powell, 1982).

Luteinizing hormone output increased progressively over three successive hourly incubations with 300 nM-PDBu and was greatest using tissue from pro-oestrous or oestrous rats. The PLA_2 inhibitor quinacrine (50 μM) significantly attenuated PDBu-induced LH release from pro-oestrous and oestrous but not metoestrous or dioestrous tissue. In contrast, the magnitude of PDBu-induced GH release was unaltered throughout the oestrous cycle and was never reduced by quinacrine. Phospholipase A_2 activity in anterior pituitary tissue was stimulated $1{\cdot}89 \pm 0{\cdot}09$-fold (mean $\pm$ S.E.M., $n = 8$) by 300 nM-PDBu. These results indicate that activation of PLA_2 by PKC can occur in some pituitary cells and may have a role in influencing hormone release from particular cell types.

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Differential activation of phospholipase A_2 by protein kinase C in pituitary cells

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Phorbol esters have been shown to induce secretion of luteinizing hormone (LH) and growth hormone (GH) from anterior pituitary tissue *in vitro* [1] implicating a role for protein kinase C (PKC) in triggering and/or modulating release of these hormones. Using pituitary tissue from pro-oestrous female rats, we have previously shown that phorbol 12,13-dibutyrate (PDBu) induced LH release is sensitive to the PKC inhibitors staurosporine and H7 [1]. In contrast, PDBu-induced GH release is sensitive to staurosporine only, thus suggesting that pharmacologically distinct forms of PKC (perhaps different structural isoforms) are involved in the modulation of LH release rather than GH release. Further observations that the temporal pattern of phorbol induced-LH release differs from that of GH release led us to consider that PKCs might exert actions on different targets in gonadotrophes from those in somatotrophes to control hormone secretion. Since arachidonic acid (AA) and its metabolites have been shown to induce release of pituitary hormones [2] we postulated that phospholipase A_2 (PLA_2) may be one such target for PKC in mediating pituitary hormone release. Here, we investigated the possibility that pituitary PLA_2 could be activated by PKC. The differential effects of PKC- and PLA_2-inhibitors on PDBu-induced pituitary hormone release shown here suggest: (1) that only certain forms of PKC can modulate PLA_2 activity and (2) that crosstalk between PKC and PLA_2 may be an important process in secretory responses in some but not all pituitary cell types.

Pituitary hormone release was measured using pituitary tissue obtained from pro-oestrous female cob wistar rats that had been maintained under controlled lighting and temperature. Briefly, pituitary glands were removed by 1300 h on the day of pro-oestrus and the anterior lobes removed and hemisected. Each hemisected lobe was placed into 2 ml of HEPES-buffered Minimal Essential Medium containing Earle's salts (MEM). After 30 minutes preincubation at 37°C, 95%O_2/5%CO_2 in a shaking water bath, the medium was replaced. From then onwards, medium was changed at hourly intervals with or without the appropriate combination of drugs. Medium LH and GH content were measured by radioimmunoassay.

Pituitary PLA_2 activity was determined by measuring [^{3}H]-AA release from prelabelled pituitaries. Pituitary tissue was removed from pro-oestrous rats as described above. Anterior pituitaries were quartered and placed in MEM (4 x ¼ per flask). After 30 minutes preincubation, the medium was replaced with MEM containing 0.5 μCi [^{3}H]-AA. After a 2 hour incubation period (37°C, 95% O_2 5% CO_2), the label was removed and the tissue washed twice with MEM containing 1% BSA. Tissue was then incubated for a further hour in MEM containing 0.5% BSA with the appropriate combination of drugs. Release of [^{3}H]-AA was measured by extraction of the incubation medium with octadecyl silica. Fractions containing lipids and metabolic derivatives were sequentially eluted with the solvents described by Powell (1982) [3]. The fraction taken to represent [^{3}H]-AA released from the tissue contained over 83% of authentic AA that was added subsequent to tissue incubation and carried through the extraction procedure.

Luteinizing hormone release from pro-oestrous anterior pituitary tissue progressively increased over 3 consecutive hourly incubations with PDBu (300 nM) from a level of 4.3±0.8 ng/ml (mean ±SEM,n=4) in the basal hour to 29.3±4.3ng/ml,n=4 in the third hour of incubation with PDBu. Growth hormone release induced by PDBu was maximal by the first hour of incubation increasing to a level of 3.14 ±0.31, n = 19 fold of basal in the first hour with the phorbol. In the presence of the PLA_2 inhibitor, quinacrine (50 μM), PDBu-induced LH release was significantly reduced by the 3rd hour of phorbol incubation from a level of secretion of 29.3±4.3ng/ml,n=4 in the absence of quinacrine to 13.2±0.9ng/ml,n=4 in the presence of quinacrine. Phorbol 12,13-dibutyrate-stimulated GH release was unaffected by the presence of quinacrine. Similarly, PDBu (300 nM) induced an 87 ± 8% (mean ± SEM, n = 10) increase in pituitary PLA_2 activity, an effect which was completely blocked by quinacrine (50 μM). Phorbol 12,13-dibutyrate-induced PLA_2 activity was also significantly, but only partially reduced to an increment of 48±11% (n=14) over basal activity by 30 μM H7 and was completely inhibited to a basal level of activity by 300 nM staurosporine.

The susceptibility of the PDBu-induced LH secretory response, but not the GH secretory response, to quinacrine indicates that in gonadotrophes, but not somatotrophes, a PKC might act to cause hormone release by a pathway involving increased PLA_2 activity. The evidence that PLA_2 may have a role in mediating phorbol-induced hormone release is strengthened by the observation that PDBu stimulates an increase in pituitary PLA_2 activity. Interestingly, the susceptibility of the PKC form(s) stimulating pituitary PLA_2 activity to PKC inhibitors matches the PKC-inhibitor profile observed in induction of LH but not GH release. That is, the actions of PDBu on LH release and PLA_2 activity are staurosporine and H7 sensitive, whereas PDBu-stimulated GH release is susceptible to staurosporine only. Thus it appears to be unlikely that a PKC-mediated increase in PLA_2 activity occurs in the GH secretory response to PDBu. In contrast, in gonadotrophes, it appears that a form of PKC is present which is capable of acting to enhance PLA_2 activity and consequently evoke hormone release. These results lend support to other evidence [4,5] for pharmacological heterogeneity in the functional cellular actions of PKC(s) and further suggest that distinct forms of PKC may have selective cellular targets.

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Br. J. Pharmacol. 104, 452P

Characterisation of [^{3}H]dimethylstaurosporine binding sites by displacement studies using protein kinase C inhibitors.

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Staurosporine inhibits protein kinase C (PKC) activity by interacting with, or close to, its ATP binding site (Nakadate, *et al,* 1988). Since the ATP binding sites of different serine/threonine- and tyrosine-specific kinases show homology, staurosporine has only modest selectivity for PKC (Rüegg & Burgess, 1989) suggesting that its radiolabelled derivative, [^{3}H]N,N-dimethylstaurosporine ([^{3}H]DMS), is a useful tool for characterisation of different classes of protein kinase within a tissue. We have characterised [^{3}H]DMS binding sites in rat midbrain and lung cytosol by displacement studies using the PKC inhibitors, H7 and staurosporine.

Adult male rat midbrain or lung was homogenised in 20mM Tris-HCl (pH7.5) with 50mM EtSH ,2mM EDTA, and 1mM phenylmethylsulphonyl fluoride, then centrifuged (12,000g, 4°C, 20 mins). The supernatant was recentrifuged (12,000g, 4°C, 5mins) and this supernatant taken to represent cytosol. The binding assay constituents were 50mM Tris-HCl (pH8.0), 1µg/ml bovine gamma globulin (BGG), 1mM dithiothreitol, [^{3}H]DMS (5nM), H7 or staurosporine and cytosol (Gross, *et al,* 1990). Total binding was measured in the absence of PKC inhibitor and non-specific binding (which was approximately 25% of total binding) was defined in the presence of 3µM staurosporine. After a 30 min incubation (4°C), the protein was precipitated on ice with 1mg/ml BGG and 10% polyethyleneglycol 8000 and pelleted by centrifugation (12,000g, 4°C, 5 mins). The pellet was washed once with 1ml of ice cold Tris-HCl then counted. Staurosporine displaced specific [^{3}H]DMS binding to sites in lung and midbrain cytosol with Hill slopes of 0.58±0.07 and 0.22±0.05 respectively, consistent with both tissues having more than one site with different affinities for staurosporine. Binding to midbrain sites was more sensitive to displacement by staurosporine (IC_{50}=0.23±0.06nM) than lung sites (IC_{50}=9.38±1.93nM). Midbrain cytosol contained sites sensitive to very low concentrations of staurosporine (with 27±4% displacement at just 0.02nM), that were apparently absent in lung. The H7 displacement curves were similar in both tissues. However, the potency of H7 was quite low with concentrations up to 500µM displacing only approximately 40% of total binding.These results are consistent with both lung and midbrain cytosol containing more than one kinase type which differ in their affinities for staurosporine. Midbrain, but not lung, appears to express a kinase (or set of kinases) which is highly sensitive to staurosporine,much more so than has previously been reported for staurosporine on purified kinase activity (Gross, *et al,* 1990). However, H7 was unable to distinguish any sites unique to either tissue. The weak displacement of binding by H7 suggests that the PKC recognition sites for staurosporine and H7 are not identical.

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EVIDENCE FOR A ROLE OF PHOSPHOLIPASE A_2 IN THE MECHANISM OF LUTEINIZING HORMONE-RELEASING HORMONE PRIMING IN RAT ANTERIOR PITUITARY TISSUE

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Key words: luteinizing hormone-releasing hormone, gonadotrophin, phospholipase A_2, arachidonic acid

ABSTRACT

The phospholipase A_2 (PLA_2) inhibitors, quinacrine, aristolochic acid, ρ-bromophenacyl bromide and ONO-RS-082, blocked the priming effect of luteinizing hormone releasing hormone (LHRH), but not acute LHRH-induced gonadotrophin release measured from pro-oestrous rat anterior pituitary pieces, *in vitro*. These results suggest that the intracellular mechanisms involved in LHRH priming are distinct from those which mediate LH release. Furthermore, neither LHRH-induced LH release from pre-primed tissue nor Ca^{2+}-induced LH release were attenuated by quinacrine, indicating that this inhibitor does not interfere with the general, Ca^{2+}-dependent secretory apparatus of the gonadotroph. Luteinizing hormone-releasing hormone induced the release of [^{3}H]arachidonic acid ([^{3}H]AA) from [^{3}H]AA prelabelled pro-oestrous rat anterior pituitary tissue; a response which was sensitive to PLA_2 inhibitors. However, LHRH priming was not prevented by the inhibitors of AA metabolism, NDGA and ETYA, except at high, non-specific concentrations, suggesting that metabolism of AA may not be essential for LHRH priming. These results indicate that LHRH receptors in pro-oestrous rat anterior pituitary gonadotrophs can be coupled to PLA_2 activation and that modulation of PLA_2 activity is required for the induction of LHRH priming.

INTRODUCTION

Anterior pituitary responsiveness to luteinizing hormone-releasing hormone (LHRH) is enhanced by prior exposure of the tissue to the peptide; a phenomenon known as LHRH priming (Aiyer, Chiappa & Fink, 1974). Consequently, after an initial exposure to LHRH, stimulus-induced luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release are both greatly enhanced. Although LHRH priming is important for the timing and induction of pre-ovulatory surge of LH (Fink, 1988), little is known about the intracellular mechanism by which it occurs. However, the priming action of LHRH appears to be distinct from the mechanism by which the peptide induces gonadotrophin release (Pickering & Fink, 1976; Fink, 1988; Johnson, Mitchell & Thomson, 1992). For example, LHRH priming, but not initial secretory responses to LHRH may be dependent upon protein synthesis and gonadotroph microfilament integrity (Pickering & Fink, 1979). Recent evidence from our laboratory has suggested that LHRH priming involves a facilitation of stimulus-secretion coupling in the gonadotroph. Luteinizing hormone-releasing hormone receptor activation is associated with phosphoinositide hydrolysis (Schrey, 1985; Andrews & Conn, 1986) and production of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which releases Ca^{2+} from intracellular stores, and diacylglycerol (DAG), which activates protein kinase C (PKC). Priming is accompanied by a potentiation of LHRH-induced $Ins(1,4,5)P_3$ production and Ca^{2+} mobilisation (Mitchell, Johnson, Ogier *et al.* 1988) and may be dependent upon the activation of a distinct form of PKC, or a closely related kinase (Johnson *et al.* 1992).

Several lines of evidence suggest that activation of phospholipase A_2 (PLA_2) is also important for responses to LHRH. The release of LH can be induced from primary cultures of rat gonadotrophs by the PLA_2 activator,

melittin, and by exogenously added PLA_2 (Kiesel, Rabe, Hauser *et al.* 1985). In addition, LHRH induces the release of arachidonic acid (AA), a product of PLA_2 action, and AA metabolites (Vanderhoek, Kiesel, Naor *et al.* 1984; Catt, Loumaye, Wynn *et al.* 1985) and exogenously added AA can induce LH release from dispersed anterior pituitary cells (Naor & Catt, 1981; Naor, Vanderhoek, Lindner *et al.* 1983; Hulting, Lingren, Hökfelt *et al.* 1984; Kiesel, Przylipiak, Emig *et al.* 1987) suggesting that this fatty acid may have a second messenger function in gonadotrophs.

To investigate for any possible role of PLA_2 in LHRH receptor responses in pro-oestrous rat anterior pituitary gonadotrophs, we examined the effects of a number of PLA_2 inhibitors on LHRH-induced gonadotrophin release and on LHRH priming. Here we report that the induction of LHRH priming, but not initial secretory responses to LHRH, in pro-oestrous rat anterior pituitary tissue, *in vitro,* is dependent upon activation of PLA_2.

MATERIALS AND METHODS

Animals

Adult female COB Wistar rats (200 g - 250 g body weight; Charles River UK Ltd) were maintained under controlled lighting and temperature with free access to food pellets (CRM, Labsure, Manea, Cambs, UK) and tap water. Vaginal smears were examined and rats with a minimum of two regular oestrous cycles were anaesthetised with sodium pentabarbitone (30 mg/kg; Sagatal; May and Baker Ltd Dagenham, Essex) by 11.30 am pro-oestrus.

Chemicals

Luteinizing hormone-releasing hormone, quinacrine dihydrochloride, (Sigma Chemical Co, Poole, Dorset, UK) 1-(5-

isoquinolinesulphonyl-2 methylpiperazine) dihydrochloride (H7) (Gibco Brl, Paisley, Scotland) and aristolochic acid sodium salt (Biomol Research Laboratories, c/o Semat, St Albans, Herts, UK) were made up as stock solutions in distilled H_2O. Ionomycin, staurosporine (Novabiochem, Nottingham, UK), 4-chloro-N-(*p*-pentylcinnamoyl) anthraniric acid (ONO-RS-082) (a gift from ONO Pharmaceuticals, Osaka, Japan) and nordihydroguaiaretic acid (NDGA) (Sigma) were made up as stock solutions in dimethylformamide (DMF). Arachidonic acid, arachidic acid, *p*-bromophenacyl bromide (BrPheBr), 4, 8, 11, 14-eicosatetraynoic acid (ETYA) were purchased from Sigma and were made up as stock solutions in ethanol. The maximum concentration of DMF or ethanol (0.05 % v/v) was used in control experiments and had no effect on either baseline LH or FSH release or [^{3}H]AA release. [5, 6, 8, 9, 11, 12, 13, 15-^{3}H(N)]-Arachidonic acid (specific activity = 240 Ci/mmol) was purchased from Dupont, Dreieich, Germany.

Hormone Release Experiments

The methods used were based upon those described by Pickering and Fink (1976). Anterior pituitary glands were hemisected and each hemipituitary placed into a flask containing 2 ml of prewarmed (37°C) and gassed (95%O_2/5%CO_2) Hepes-buffered minimal essential medium (MEM) (Gibco Brl, Paisley, UK) with Earles' salts. After a 30 minute pre-incubation period in a shaking water bath (37°C, 95%O_2/5%CO_2), and every subsequent hour thereafter, the medium was replaced. In the initial, basal hour, the medium contained either a PLA_2 inhibitor or no drug. For each subsequent hour (1st h, 2nd h, 3rd h), medium also contained either LHRH (1 nmol/l) or ionomycin (30 µmol/l) or arachidonic acid (300 µmol/l) or arachidic acid (300 µmol/l).

The medium removed at the end of each incubation period was kept at -20°C until it was radioimmunoassayed for LH and FSH (Niswender, Midgley, Monroe *et al.* 1968; Daane and Parlow, 1971). The standards used were NIH-LH-S18 for the data in Figure 1a and NIADDK-rat LH-RP2 for the remaining data and NIADDK-rat-FSH-RP2.

[^{3}H]Arachidonic Acid Release Measurements

To obtain an index of anterior pituitary PLA_2 activity, [^{3}H]AA release was measured from pre-labelled tissue as follows. Anterior pituitary glands were removed, hemisected and each hemipituitary cut into two equal quarters. Pairs of pituitary quarters were then placed into a silanised flask containing 1 ml of prewarmed (37°C) and pregassed (95%O_2/5%CO_2) MEM and preincubated in a shaking water bath (37°C, 95%O_2/5%CO_2) for 30 minutes. The medium was then replaced with fresh MEM containing 0.5 μCi of [^{3}H]AA and the tissue incubated with the label for 2 hours. The tissue was then washed three times in MEM containing 1% essential fatty acid-free bovine serum albumin (BSA; Sigma, Poole, Dorset, UK) to remove unesterified [^{3}H]AA.

For studies of [^{3}H]AA release, the tissue was incubated in MEM + 0.5% BSA (as a trap for released [^{3}H]AA) in the presence or absence of LHRH. After 15 min, the incubation medium was removed and the [^{3}H]AA released was measured by lipid extraction and reverse-phase liquid chromatography on octadecyl silica (ODS) using the solvent system described by Powell (1982). Ethanol was added to the incubation medium to give a final ethanol concentration of 30% v/v and the medium was centrifuged (10', 5°C, 3000 g). The supernatant was removed and adjusted to pH 3. The acidified medium (4 ml) was loaded onto a 1.25 cm ODS column (Sep pak C_{18} cartridge; Waters Chromatography, Watford, Hertfordshire, UK) which had been prewashed with 5 ml ethanol followed by 5 ml of

distilled H_2O. Solvents were then passed through the columns in the following order; 30% ethanol (20 ml), distilled H_2O (20 ml), petroleum ether (10 ml) and petroleum ether: $CHCl_3$ (1:1, 20 ml). The fraction taken to represent [^{3}H]AA released from the tissue (petroleum ether: $CHCl_3$) contained over 83% of authentic standard [^{3}H]AA that had been incubated with denatured tissue (previously homogenised in 30% ethanol) and carried through the extraction procedure.

The radioactivity remaining in the tissue was determined by washing the tissue twice in 1 ml MEM containing 1% BSA and the tissue was homogenised in 1 ml MEM + 0.5% BSA using a hand held tissue grinder. Ethanol was then added to the tissue homogenate to give a final concentration of 30% v/v. The homogenate was then centrifuged (10 min, 5°C, 3000 g) and the supernatant acidified to pH 3. Aliquots of the supernatants from the tissue homogenate and the incubation media were counted to give a measurement total amount of label incorporated into the tissue for each flask.

In the experiments where the effects of inhibitors were examined on LHRH responses, the tissue was preincubated for 15 minutes with the appropriate inhibitor, the medium was removed and the tissue incubated with, in addition, LHRH as described above.

Data Analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical analyses were carried out using the Mann-Whitney U-test. The concentration of PLA_2 inhibitor which prevented 50 % of the maximal response to LHRH (IC_{50} value) was obtained from fitting the data with the non-linear, error-weighted iterative curve fitting program, P. Fit (Biosoft, Cambridge, UK).

RESULTS

The effect of PLA_2 inhibitors on LHRH responses measured in pro-oestrous rat anterior pituitary tissue.

Figure 1 shows the effect of the PLA_2 inhibitor, quinacrine (50 μmol/l) (Markus & Ball, 1969; Löffler ,Bohn, Hesse *et al.* 1985) on LH (Figure 1a) and FSH (Figure 1b) release from pro-oestrous rat hemipituitaries, measured over consecutive hourly incubations with LHRH (1 nmol/l). Neither basal h, nor 1st h LHRH-induced LH release were significantly altered in the presence of quinacrine. However, 2nd h LHRH-induced LH release, where LHRH priming is expressed, was significantly inhibited by quinacrine ($p < 0.05$) to levels that were 44.5 ± 3.1 % of control. Similarly, 2nd h, but not basal h or 1st h, LHRH-induced FSH release was significantly inhibited by 50 μmol/l quinacrine (Figure 1b). Quinacrine, at concentrations up to 100 μmol/l, caused a dose-dependent inhibition of 2nd h LHRH-induced LH release to levels approaching those measured during the 1st h of LHRH stimulus, but never to basal h levels (Figure 2a). Another PLA_2 inhibitor, *p*-bromophenacyl bromide (BrPheBr) (Drenth, Enzig, Kalk *et al.* 1976) also blocked LHRH-induced release of primed LH to levels approaching those measured during the 1st h of LHRH incubation, without significantly affecting 1st hour LH release (Figure 2b). However, at BrPheBr concentrations of 50 μmol/l and above, basal h LH release was elevated above control levels, suggesting that this compound may have non-specific actions at high concentrations. Other putative inhibitors of PLA_2 activity, aristolochic acid (50 μmol/l) (Vishwanath, Fawzy & Franson, 1988) and ONO-RS-082 (10 μmol/l) (Banga, Simons, Brass *et al.* 1986) both inhibited 2nd h LHRH-induced LH release without altering either basal h or 1st h LHRH-induced LH release (Table 1). Since 2nd h, but

not 1st h, LHRH-induced LH release from pro-oestrous rat hemipituitaries was inhibited these compounds, PLA_2 activation may be required for LHRH priming , but not for initial responses to LHRH.

Neither quinacrine (100 μmol/l), ONO-RS-082 (10 μmol/l) or aristolochic acid (50 μmol/l) had any significant effect on baseline gonadotrophin release when measured over several hours of incubation.

To examine the role of PLA_2 in LHRH priming in more detail, a number of experiments were carried out. These experiments considered the critical period for the effect of quinacrine on pituitary responses and the specificity of its action (Table 2, Figure 3). Firstly, hemipituitary pieces were incubated for a basal h in medium only or with quinacrine (50 μmol/l), followed by a 1st h incubation with, in addition, LHRH (1 nmol/l). After the 1st h incubation, quinacrine was removed by washing the tissue three times in fresh pre-warmed and pre-gassed MEM and the washed tissue was incubated for a final hour (2nd h) with LHRH only. Control treatments received identical incubations with LHRH and washing, but in the absence of quinacrine. Consistent with the results shown in Figures 1 and 2, quinacrine did not inhibit either basal h or 1st h LHRH-induced LH release (Figure 3a). However, following a 1st h incubation with LHRH and quinacrine, 2nd h LHRH-induced LH release was significantly reduced in comparison to the controls, although the tissue had been washed to remove the inhibitor from the 2nd h incubation period. These results indicate that quinacrine may inhibit a PLA_2-dependent induction of LHRH priming during the 1st h of incubation, reducing 2nd h LHRH-induced LH release as a consequence. However, it is possible that the washout procedure was not completely effective and that a significant amount of quinacrine was present in the tissue during the 2nd h of LHRH incubation.

Consequently, additional experiments were carried out to further examine the effects of quinacrine on the induction of LHRH priming

In a second experiment, the effect of quinacrine was examined on LHRH-induced LH release from pituitary tissue which had been previously primed (Figure 3b). Hemipituitaries were incubated for a 1st h with LHRH (1 nmol/l) followed by a further 2 consecutive hourly incubations with LHRH, either in the presence or absence of quinacrine (50 μmol/l). Quinacrine had no significant inhibitory action on LHRH-induced LH release from pre-primed tissue, even over two hours of incubation which would allow for any possible slow-developing inhibitory effect on the mechanism of hormone release.

To test the actions of quinacrine on the secretory apparatus of the gonadotroph, the effects of this drug were examined on LH release induced by raising intracellular Ca^{2+} levels (Table 2). In the presence of ionomycin (30 μmol/l), LH release was increased above basal h levels, but with a temporal pattern that did not exhibit 'priming'. Quinacrine (50 μmol/l) had no significant inhibitory effect on ionomycin-induced LH release measured during any hour of incubation indicating that this drug does not inhibit the general secretory apparatus of the gonadotroph. In addition, these results suggest that PLA_2 is not involved in the mechanism of Ca^{2+}-induced LH release. In contrast, ionomycin-induced LH release from tissue which had been previously primed by a 1st h incubation with LHRH was significantly inhibited when quinacrine was present during the LHRH incubation period.

In summary, PLA_2 inhibitors prevented LHRH responses only when present during the time when the induction of LHRH priming takes place i.e. the 1st h of LHRH treatment (Figures 1, 2, 3 and Table 2). In addition, quinacrine did not inhibit the actions of ionomycin; a secretagogue that

does not elicit a priming response. These results therefore indicate an involvement of PLA_2 in the induction, but not in the expression of LHRH priming.

The effect of LHRH on pro-oestrous rat anterior pituitary PLA_2 activity.

The influence of LHRH on gonadotroph PLA_2 activity was examined, more directly, by measuring [^{3}H]AA release from pre-labelled pro-oestrous rat anterior pituitary tissue. After a 15 min incubation with LHRH (1 nmol/l), [^{3}H]AA release increased by approximately 2 fold over basal levels (Table 3). An increase in the release of free [^{3}H]AA levels from cellular phospholipids may result either from the action of PLA_2 or by the sequential actions of phospholipase C (PLC), which releases DAG, and DAG lipase, which will catalyse the removal of a fatty acid from the glycerol backbone of DAG (Naor, 1991). To determine the pathway of LHRH-induced [^{3}H]AA release from pro-oestrous rat anterior pituitary tissue, we examined the effect of quinacrine and the DAG lipase inhibitor, RHC 80267, on the LHRH response. The inhibitors were used at concentrations reported to inhibit the activity of their targets with minimum side effects (Hoffman, Prescott and Majerus, 1982; Sutherland and Amin, 1982). This response was almost completely prevented by quinacrine (50 μmol/l), but not by RHC 80267 (80 μmol/l), suggesting that the main route of [^{3}H]AA release induced by LHRH is via a pathway involving PLA_2 but not DAG lipase activation.

We have previously shown that the induction of LHRH priming is dependent upon the activation of a pharmacologically distinct PKC (or closely related kinase) which is readily inhibited by the PKC inhibitors, staurosporine and Ro 31-8220, but not by another PKC inhibitor, H7 (Johnson *et al.* 1992). To test for the possible involvement of this PKC-like kinase in the mechanism of LHRH-induced activation of PLA_2 , the effects

of H7 and staurosporine were examined on LHRH-induced [^{3}H]AA release (Table 4). [^{3}H]Arachidonic acid release induced by LHRH was readily blocked by staurosporine at a concentration (300 nmol/l) which inhibits LHRH priming (Johnson *et al.* 1992). In contrast, LHRH-induced [^{3}H]AA release was relatively resistant to block by H7 at a concentration (30 μmol/l) which is usually associated with the inhibition of PKC action in other cell types (Hidaka, Inagaki, Kawamoto *et al.* 1984), but which is unable to inhibit LHRH priming (Johnson *et al.* 1992). Thus, the induction of LHRH priming may occur by a sequence of events which involve activation of an H7-resistant form of a PKC-like kinase and its subsequent action to increase PLA_2 activity.

The involvement of arachidonic acid and arachidonic acid metabolites in LHRH responses

Since PLA_2 activation may be required for the induction of LHRH priming, and LHRH induces an increase in [^{3}H]AA release, AA may be a mediator of LHRH action. In the presence of AA (300 μmol/l), LH release from pro-oestrous hemipituitaries was significantly increased during the 2nd and 3rd hs of incubation (Figure 4a). However, the saturated congener of AA, arachidic acid (300 μmol/l), which lacks many of the biological actions of AA (Beaumier, Faucher & Naccache, 1987; Negishi, Ito & Hayaishi, 1990), also enhanced LH release (Figure 4b), suggesting that the direct hormone releasing effect of AA on gonadotrophs is non-specific.

Since previous reports have suggested that lipoxygenase and epoxygenase metabolites of AA may modulate gonadotrophin release (Naor *et al.* 1983; Snyder, Capdevila, Chacos *et al.* 1983; Catt *et al.* 1985; Kiesel *et al.* 1987), we examined the effects of lipoxygenase inhibitors on LHRH responses in pro-oestrous rat hemipituitary pieces. At a concentration of 10 μmol/l, where ETYA is reported to lipoxygenase and cyclo-oxygenase

pathways of AA metabolism (Hamberg and Samuelsson, 1974), LHRH-induced LH release was unaltered during any hour of incubation (Table 5) suggesting that AA metabolism may not be required for LHRH responses in pro-oestrous rat hemipituitaries. At a higher concentration, ETYA (30 μmol/l) inhibited 2nd h, but not 1st h, LHRH-induced LH release to levels that were not significantly different from basal h measurements. Although ETYA can inhibit the epoxygenase pathway at this higher concentration (Capdevila, Gil, Orellana *et al.* 1988), ETYA, at this level, can also block PLA_2 activity (Lanni and Becker, 1985). Nordihydroguaiaretic acid, when used at a concentration (10 μmol/l) reported to inhibit the epoxygenase and lipoxygenase pathways (Capdevila *et al.* 1988; Smith and Bowman, 1982) had no effect on LHRH responses during any hour. However, when used at a concentration of 30 μmol/l, NDGA inhibited both 1st and 2nd h LHRH-induced LH release to baseline levels (Table 5), but at this level, ETYA can also inhibit PLA_2 activity (Lanni and Becker, 1985). Neither ETYA (10 μmol/l) nor NDGA (10 μmol/l) had any significant effect on baseline LH release measured over several hours of incubation.

DISCUSSION

Previous reports suggest that stimulation of rat gonadotroph and rat granulosa cell LHRH receptors can lead to activation of PLA_2 (Naor & Catt, 1981; Minegishi, Wang & Leung, 1987). The present evidence suggests that, in pro-oestrous rat gonadotrophs, activation of PLA_2 may be required for the induction of LHRH priming, but not for initial LHRH-induced gonadotrophin release. Quinacrine, BrPheBr, ONO-RS-082 and aristolochic acid, blocked 2nd h LHRH-induced release of primed LH, but not 1st h LHRH-induced LH release (Figures 1, 2 and Table 1), with potencies similar to their effects on PLA_2-mediated responses in other cell types (Löffler *et al.* 1985; Banga *et al.* 1986; Rosenthal, Vishwanath & Franson, 1989; Tohmatsu, Nakashima & Nozawa, 1989). Notably, the concentration of quinacrine which was reported to inhibit later secretory responses to LHRH in primary cultures of anterior pituitary cells (IC_{50} = 20 μmol/l (Naor and Catt, 1981)) is within the range described here for the block of LHRH priming (IC_{50} = 34.0 ± 10.3 μmol/l). The 2nd h response to LHRH was blocked by these drugs to quantities that approached 1st h levels of LHRH-induced LH release, but never to baseline levels. Thus, PLA_2 activation may be required for enhanced gonadotroph responsiveness. Consistent with this hypothesis, LHRH-induced LH release was only inhibited if quinacrine was present throughout the time when LHRH priming occurs i.e. the 1st h of LHRH incubation, whereas LHRH-induced LH release from pre-primed tissue was not blocked by quinacrine (Figure 3). Therefore, the induction, but not the expression, of priming may be dependent upon PLA_2 activation. Quinacrine did not appear to inhibit primed LH release by affecting the later stages in the mechanism of gonadotrophin release since this drug did not alter ionomycin-induced LH release. However, if the tissue was primed by

exposure to LHRH, facilitated LH release in response to the Ca^{2+}-ionophore was blocked by quinacrine to 'non-primed' levels (Table 2).

The specificity of both quinacrine and BrPheBr as inhibitors of PLA_2 has been questioned in a number of studies (Irvine, 1982; Chang, Musser & McGregor, 1987). High concentrations of quinacrine may perturb membrane architecture and alter cell function (Dise, Burch & Goodman, 1982). However, quinacrine did not alter Ca^{2+}-induced LH release (Table 2) suggesting that a non-specific action of this drug is unlikely to be responsible for its effects on LHRH responses. Bromophenacyl bromide inhibits PLA_2 activity by forming a covalent bond with a histidine residue which is situated near the Ca^{2+}-binding site of the enzyme (Volwerk, Pieterson & de Haas, 1974; Drenth *et al.* 1976; Roberts, Deems, Mincey *et al.* 1977), but can covalently modify many other proteins (Erlanger, Vratsanos, Wassermann *et al.* 1965) which may account for the effects of high concentrations of BrPheBr on baseline LH release. Although BrPheBr and quinacrine are putative inhibitors of PLC activity (Hofmann *et al.* 1982), these compounds were unable to prevent the presumed PLC-dependent, 1st h of LHRH-induced LH release. Furthermore, at the concentrations used here, these compounds have been reported to inhibit PLA_2 in other systems without affecting PLC activity (Lazarewicz, Wroblewski, Palmer *et al.* 1988). In agreement with the actions of quinacrine and BrPheBr on LHRH responses, another PLA_2 inhibitor, ONO-RS-082, blocked the primed response to LHRH, at a concentration which can inhibit PLA_2 activity in platelets, without affecting PLC activity (Tohmatsu *et al.* 1989; Banga *et al.* 1986).

In agreement with the pharmacological evidence presented here which suggests that LHRH receptors modulate PLA_2 activity, stimulation of anterior pituitary pieces with LHRH is accompanied by a relatively rapid (15

min) increase in [^{3}H]AA; a response which was blocked by inhibitors of PLA_2, but not DAG lipase action (Table 3). However, this result contrasts with a previous report where RHC 80267, at concentrations that block DAG lipase activity with little effect on PLA_2 action, inhibited approximately 40 % of LHRH-induced LH release from dispersed anterior pituitary cells (Chang, Morgan & Catt, 1988). The reason why DAG lipase is apparently involved in LHRH responses in dispersed anterior pituitary cells, but not in pro-ostrous rat anterior pituitary tissue is uncertain, but may reflect an alteration in gonadotroph function following cell dispersal and culture.

In the LHRH receptor signal, modulation of gonadotroph PLA_2 activity may be dependent upon the activity of PKC, suggesting that PLA_2 activation may occur down-stream from inositol phospholipid hydrolysis. An initial indication of a possible link between PLA_2 and PKC activation in response to LHRH came from the observation that activation of both of these enzymes is required for LHRH priming (see also Johnson *et al.* 1992). In addition, staurosporine, but not H7, inhibited LHRH-induced [^{3}H]AA release at concentrations which are equivalent to their effects on LHRH self-priming (IC_{50} = 26.3 ± 7.0 nmol/l and 71.6 ± 13.3 μmol/l for staurosporine and H7 respectively) (Johnson *et al.* 1992). Thus, LHRH receptor-mediated activation of an H7-resistant, PKC-like kinase may modulate PLA_2 activity, enhancing gonadotroph responsiveness.

Although the product of PLA_2-catalysed phospholipid hydrolysis, AA could induce LH release, the saturated congener, arachidic acid, could also elicit the same effect (Figure 4). Arachidic acid lacks many of the biological actions of AA. For example, AA, but not arachidic acid, can induce Ca^{2+} release from intracellular stores (Beaumier *et al.* 1987) and can induce inositol phospholipid hydrolysis (Negishi *et al.* 1990). It seems likely that AA and arachidic acid may induce LH release by a non-specific action,

perhaps by having a detergent-like effect on the gonadotroph cell membrane (Karnovsky, Kleinfeld, Hoover *et al.* 1982; Kaye, van der Merwe, Miller *et al.* 1992). In other cell types, endogenous AA production can be dissociated from exocytosis (Churcher, Allan & Gomperts, 1990; Morgan & Burgoyne, 1990) suggesting that AA is not a direct mediator of secretion. In cell-free systems, however, AA can enhance fusion of secretory granules into membranes in a Ca^{2+}-dependent manner and may, therefore, modulate exocytosis (Creutz, 1981). The possibility that an equivalent action of AA in gonadotrophs may enhance Ca^{2+}-induced LH release, thereby contributing to the LHRH priming effect, is an interesting prospect.

The LHRH priming affect may not be dependent upon lipoxygenase metabolites of AA since neither ETYA nor NDGA had any effect on LHRH responses when either compound was used at a concentration (10 μmol/l) which is said to inhibit lipoxygenase activity (Hamberg and Samuelsson, 1974; Capdevila *et al.* 1988; Smith and Bowman, 1982) (Table 5). At a higher concentration (30 μmol/l), ETYA and NDGA did inhibit priming, but at this concentration, both drugs also block PLA_2 activity (Lanni & Becker, 1985). We could therefore find no evidence at all that lipoxygenase metabolites of AA have a role in LHRH responses in pro-oestrous rat anterior pituitary tissue. However, both ETYA and NDGA can inhibit LHRH-induced LH release from primary cultures of anterior pituitary cells (Naor *et al.* 1983; Catt *et al.* 1985) and certain lipoxygenase metabolites can induce LH release from dispersed anterior pituitary cells (Naor *et al.* 1983; Catt *et al.* 1985; Kiesel *et al.* 1987). Furthermore, selective leukotriene receptor antagonists can partially inhibit LHRH-induced LH release from dispersed rat anterior pituitary cells (Catt *et al.* 1985; Kiesel, Przylipiak, Habenicht *et al.* 1991). The discrepancy between these reports and the results presented here may be accounted for by differences in the model of pituitary function chosen in

each study (see Conn, Hawes & Janovick, 1992 for review). Therefore, it is possible that lipoxygenase products have a different function in LHRH receptor-signalling in pro-oestrous rat pituitary pieces than in dispersed pituitary cells.

Epoxygenase metabolites have also been suggested to have a role in LH release responses (Snyder *et al.* 1983). However, NDGA, at a concentration reported to inhibit cyclo-oxygenase activity (10 μmol/l) (Capdevila *et al.* 1988), had no significant inhibitory effect on LHRH priming, suggesting that epoxygenase metabolites are not involved in this response. Further studies using recently developed selective inhibitors of the epoxygenase and lipoxygenase enzymes will help to clarify the role of these pathways of AA metabolism in LHRH responses in pro-oestrous rat anterior pituitary tissue.

In summary, these experiments show that the induction of LHRH-priming requires PLA_2 activation. The LHRH receptor controlled PLA_2 activity is likely to be related to the hormonally-regulated, cytosolic forms which have been described in other cell types (Gronich, Bonventre & Nemenoff, 1990). Although these PLA_2 forms can associate with the cell membrane in a Ca^{2+}-dependent manner, raised Ca^{2+} levels alone do not seem to be sufficient for full activation of the gonadotroph PLA_2, since Ca^{2+}-induced LH release was not blocked by PLA_2 inhibitors. Instead, the activity of an H7-resistant PKC is apparently required, in addition, to fully elicit gonadotroph PLA_2 activation.

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FIGURE 1

The effect of quinacrine on LHRH-induced LH (a) and FSH (b) release from pro-oestrous rat anterior pituitary tissue.

Tissue was incubated for consecutive hours. In the basal h, tissue was incubated in either medium only (open bars) or with quinacrine (50 μmol/l) (hatched bars) then, in addition, with LHRH (1 nmol/l) throughout the following 1st and 2nd h incubations. Quinacrine had no significant effect on either basal or 1st h gonadotrophin release, but significantly inhibited 2nd h, LHRH-primed LH and FSH release (*$p < 0.05$). Data are means ± S.E.M. for 4 - 6 determinations.

FIGURE 2

Concentration-response curves for the effect of (a) quinacrine and (b) ρ-bromophenacyl bromide on acute LHRH-induced LH release and LHRH priming in pro-oestrous rat anterior pituitary tissue.

Tissue was incubated for a basal h (●) in medium only or with increasing concentrations of either quinacrine (10 - 100 μmol/l) or ρ–bromophenacyl bromide (BrPheBr) (1 - 100 μmol/l) followed by 2 further consecutive hourly incubations (1st h (■) and 2nd h(▲)) with, in addition, LHRH (1 nmol/l). Both quinacrine and BrPheBr dose-dependently inhibited 2nd h LHRH primed release of LH (*$p < 0.05$). Data are means ± S.E.M. for 4 - 6. determinations.

FIGURE 3

Effect of quinacrine on the development of LHRH priming in pro-oestrous rat anterior pituitary tissue.

Hemipituitary pieces were incubated for consecutive hours (basal h, 1st h, 2nd h, 3rd h). In part a, tissue was incubated for a basal h with quinacrine (50 μmol/l) (hatched bars), or with no drug (open bars), followed by a 1st h with, in addition, LHRH (1 nmol/l). The tissue was then washed in MEM and all treatments were incubated with LHRH only during the final 2nd h (open bars). Second h LHRH-induced LH release was significantly inhibited (*$p < 0.05$) in the quinacrine treated samples. In part b, tissue was incubated for a basal h in medium only followed by a 1st h with 1 nmol/l LHRH (open bars), during which LHRH priming takes place. Throughout the 2nd and 3rd hs, tissue was then incubated with LHRH only (open bars) or with LHRH and quinacrine (50 μmol/l) (hatched bars). Quinacrine had no significant inhibitory effect on LHRH-induced LH release from pre-primed tissue. Data are means ± S.E.M. for 6 determinations.

FIGURE 4

Effect of (a) arachidonic acid and (b) arachidic acid on LH release from pro-oestrous rat anterior pituitary pieces.

Hemipituitary pieces were incubated for a basal h in medium only followed by 3 consecutive hourly incubations (1st h, 2nd h, 3rd h) either in medium only (open bars) or with (a) arachidonic acid (300 μmol/l) or (b) arachidic acid (300 μmol/l). Luteinizing hormone release was significantly increased above baseline during the 2nd and 3rd h of incubation with each fatty acid (*$p < 0.05$). Values are means ± S.E.M. for 4 or 5 determinations.

TABLE 1

The effect of PLA_2 inhibitors on initial LHRH-induced LH release and LHRH priming in pro-oestrous rat anterior pituitary tissue.

Hemipituitaries were incubated, *in vitro*, for 3 hours. In the basal h there was medium alone or a PLA_2 inhibitor (ONO-RS-082 (10 μmol/l), or aristolochic acid (50 μmol/l)). In the 1st and 2nd hs there was, in addition, LHRH (1 nmol/l). The statistical significance of the inhibitory actions of the PLA_2 inhibitors tested was determined (*$p < 0.05$). Values given are the mean ± S.E.M. with the number of determinations in parentheses.

	LH release (μg/l)		
	basal h	1st h	2nd h
LHRH	6.9 ± 2.0 (5)	24.2 ± 3.9 (5)	90.4 ± 8.8 (5)
LHRH + ONO-RS-082	5.1 ± 0.9 (4)	16.1 ± 2.4 (4)	*20.1 ± 1.6 (4)
LHRH + aristolochic acid	2.9 ± 0.6 (4)	23.4 ± 2.9 (4)	*54.9 ± 9.7 (4)

TABLE 2

The effect of quinacrine on ionomycin-induced LH release from pro-oestrous rat anterior pituitary tissue.

Hemipituitary pieces were incubated for consecutive hours. In the basal h there was medium only or quinacrine (50 μmol/l). In the following hours, in addition, the incubation medium contained ionomycin (30 μmol/l, I_1) or LHRH (1 nmol/l, L_1), followed by a 2nd h incubation with ionomycin (I_2). The statistical significance of the inhibitory effect of quinacrine was determined (*$p < 0.05$). All values are means ± S.E.M. and the number of determinations are shown in parentheses.

	LH release (μg/l)		
	basal h	1st h	2nd h
I_1 - I_2	4.5 ± 1.0 (5)	26.7 ± 2.0 (5)	26.8 ± 2.8 (5)
I_1 - I_2 + quinacrine	7.9 ± 1.3 (4)	27.5 ± 3.1 (4)	25.7 ± 3.0 (4)
L_1 - I_2	7.6 ± 0.4 (5)	28.1 ± 9.1 (5)	75.1 ± 1.0 (5)
L_1 - I_2 + quinacrine	7.5 ± 1.4 (4)	31.1 ± 7.1 (4)	*30.8 ± 1.4 (4)

TABLE 3

The effect of quinacrine and RHC 80267 on LHRH-induced [^{3}H]arachidonic acid release from prelabelled pro-oestrous rat anterior pituitary pieces.

Pairs of [^{3}H]AA prelabelled anterior pituitary pieces were preincubated for 15 minutes in medium only, or with quinacrine (50 μmol/l) or RHC 80267 (80 μmol/l). The medium was discarded and replaced with fresh medium containing either no drug (basal) or LHRH (1 nmol/l) only or LHRH together with either quinacrine or RHC 80267 and after a second 15 min incubation, [^{3}H]AA release was determined. In the presence of LHRH only, [^{3}H]AA release was increased to levels that were significantly greater than baseline (†$p<0.01$). The statistical significance of the effect of quinacrine and RHC 80267 on responses to LHRH was determined (*$p < 0.05$). Values shown are the means ± S.E.M. and the number of determinations are shown in parentheses.

	[^{3}H]AA release (% of total label incorporated)
baseline	0.85 ± 0.06 (8)
LHRH	†2.17 ± 0.16 (8)
LHRH + quinacrine	*0.92 ± 0.07 (6)
LHRH + RHC 80267	1.66 ± 0.18 (4)

TABLE 4

The effect of PKC inhibitors on LHRH-induced [^{3}H]arachidonic acid release from prelabelled pro-oestrous anterior pituitary pieces.

Pairs of prelabelled anterior pituitary quarters were preincubated for 15 minutes with either no drug, or H7 (30 μmol/) or staurosporine (300 nmol/l). The medium was replaced and fresh medium contained either no drug (baseline), LHRH (1 nmol/l), LHRH and H7 or LHRH and staurosporine. In the presence of LHRH only, [^{3}H]AA release was increased to levels that were significantly greater than baseline (†$p<0.05$). Staurosporine, but not H7, significantly inhibited LHRH-induced [^{3}H]AA release (*$p< 0.05$). Values shown are the means ± S.E.M. and the number of determinations are shown in parentheses.

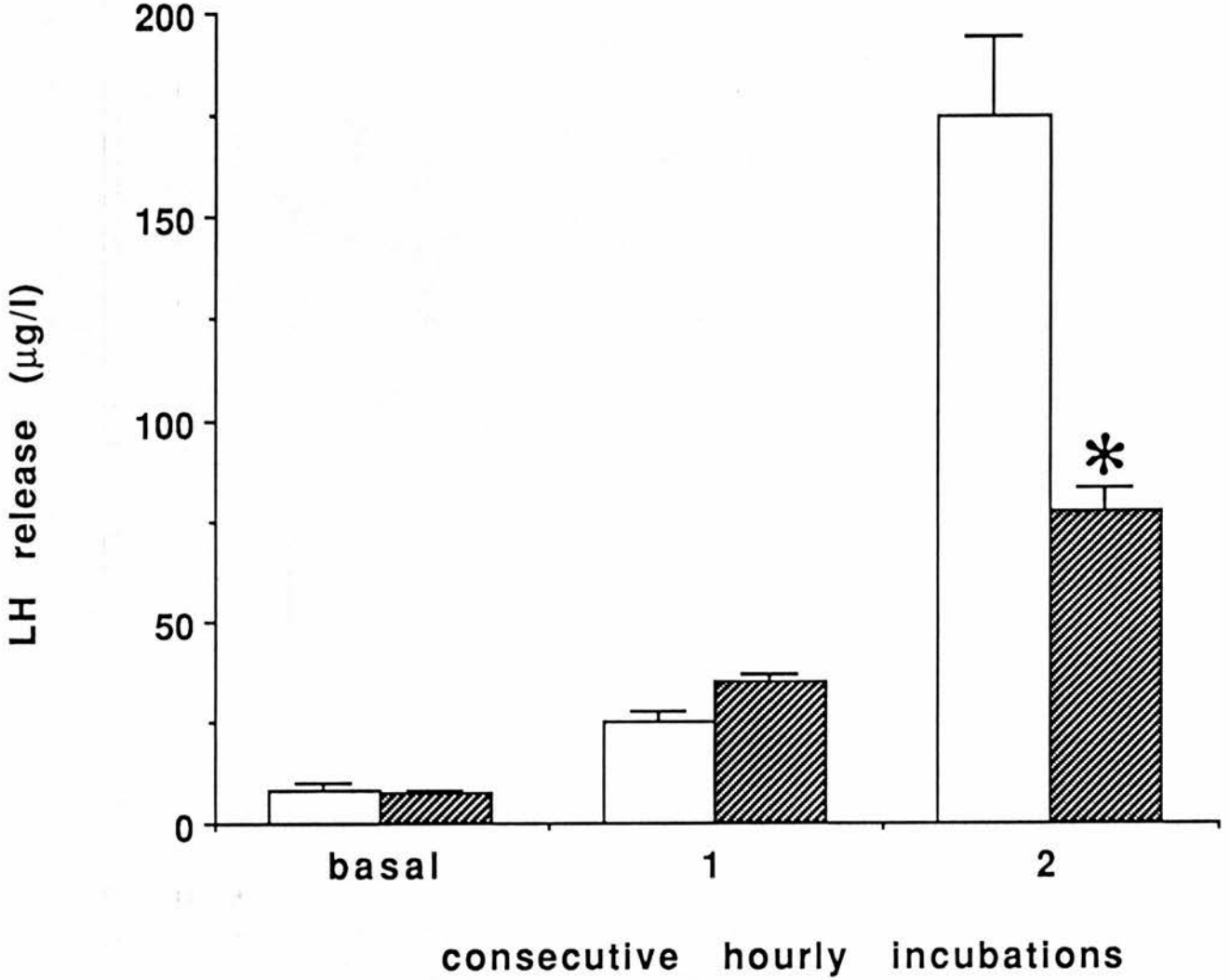
	[^{3}H]AA release (% of total label incorporated)
baseline	1.03 ± 0.09 (10)
LHRH	†2.33 ± 0.30 (10)
LHRH + staurosporine	*0.95 ± 0.04 (10)
LHRH + H7	1.79 ± 0.07 (8)

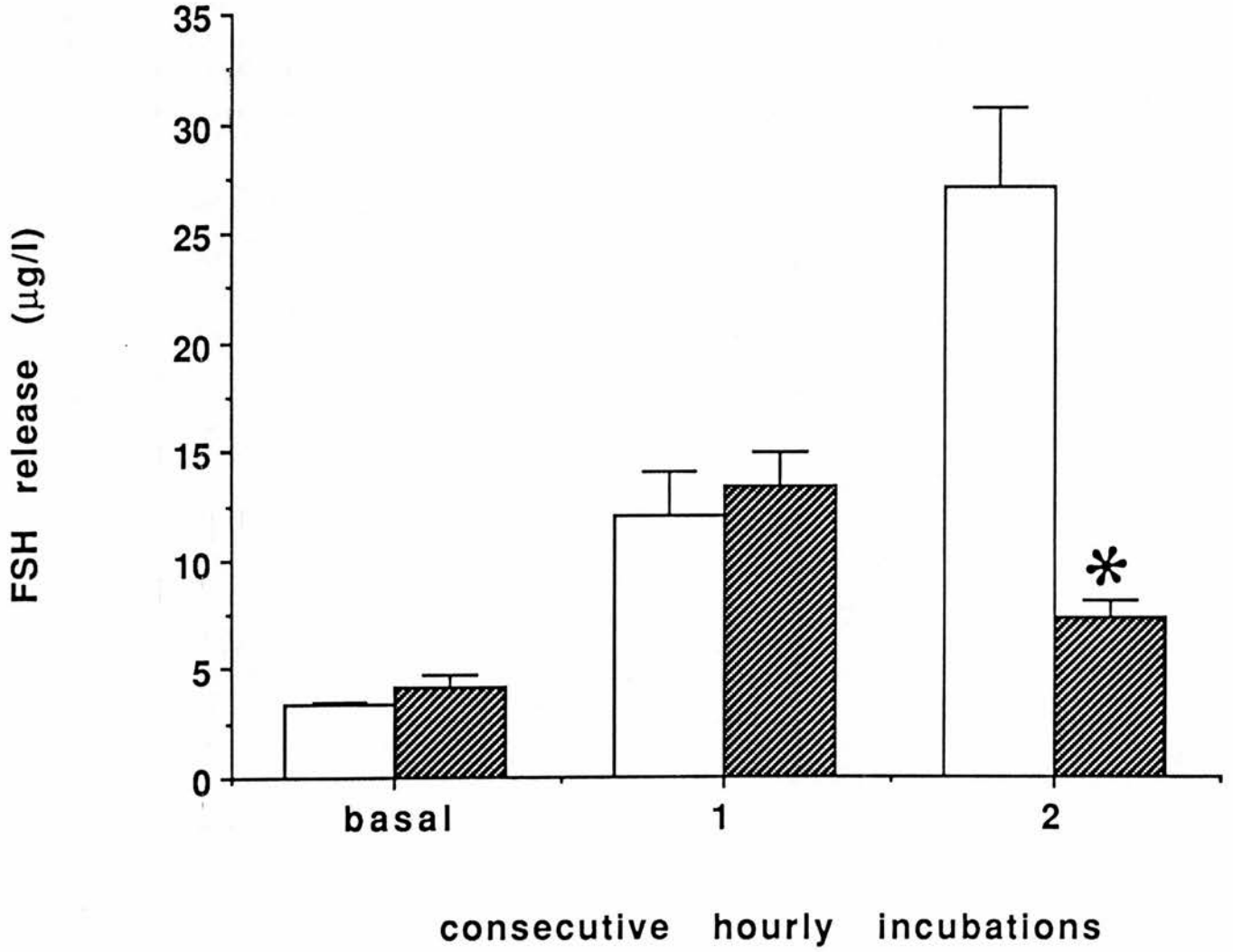
TABLE 5

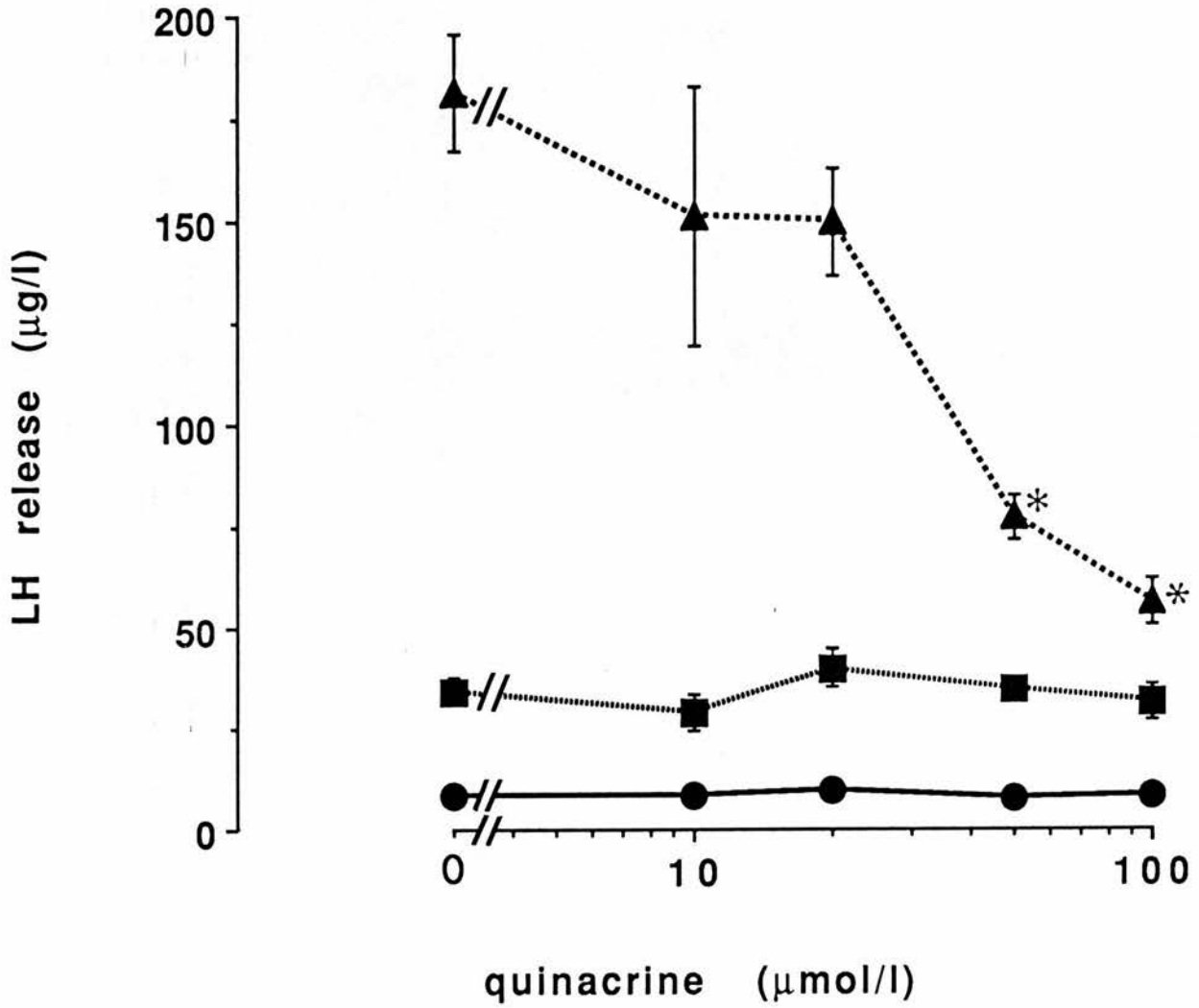
The effect of inhibitors of arachidonic acid metabolism on LHRH responses measured in pro-oestrous rat anterior pituitary.

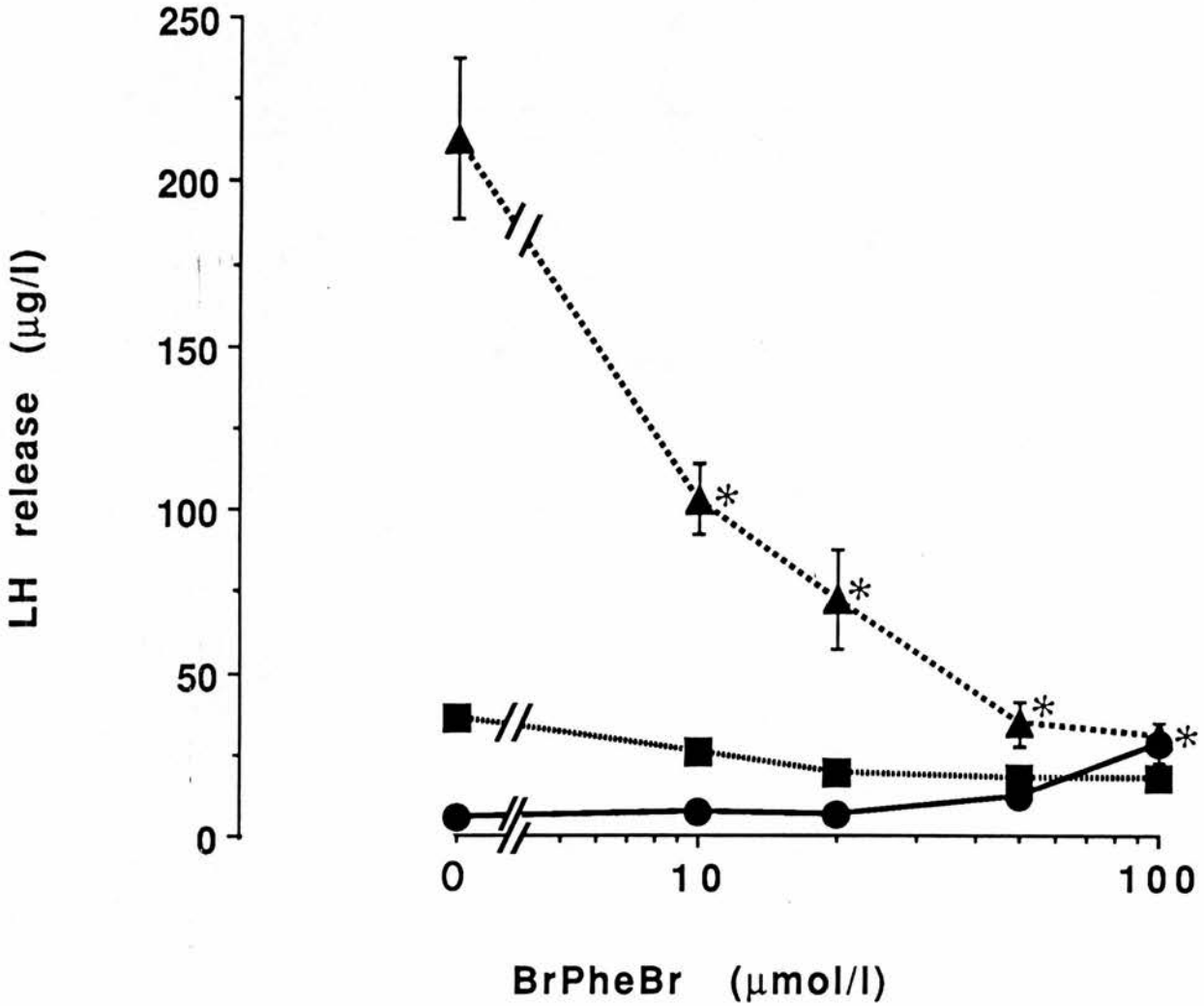
Hemipituitaries were incubated, *in vitro*, for consecutive hours. In the basal h, tissue was incubated in medium only, or in the presence of ETYA, (10 or 30 μmol/l) or NDGA, (10 or 30 μmol/l). In the following hours (1st h, 2nd h), the incubation medium contained, in addition, LHRH (1 nmol/l). The statistical significance of the effects of each inhibitor was determined ($p < 0.05$). Values shown are for means ± S.E.M. and the number of replicates are shown in parenthesis.

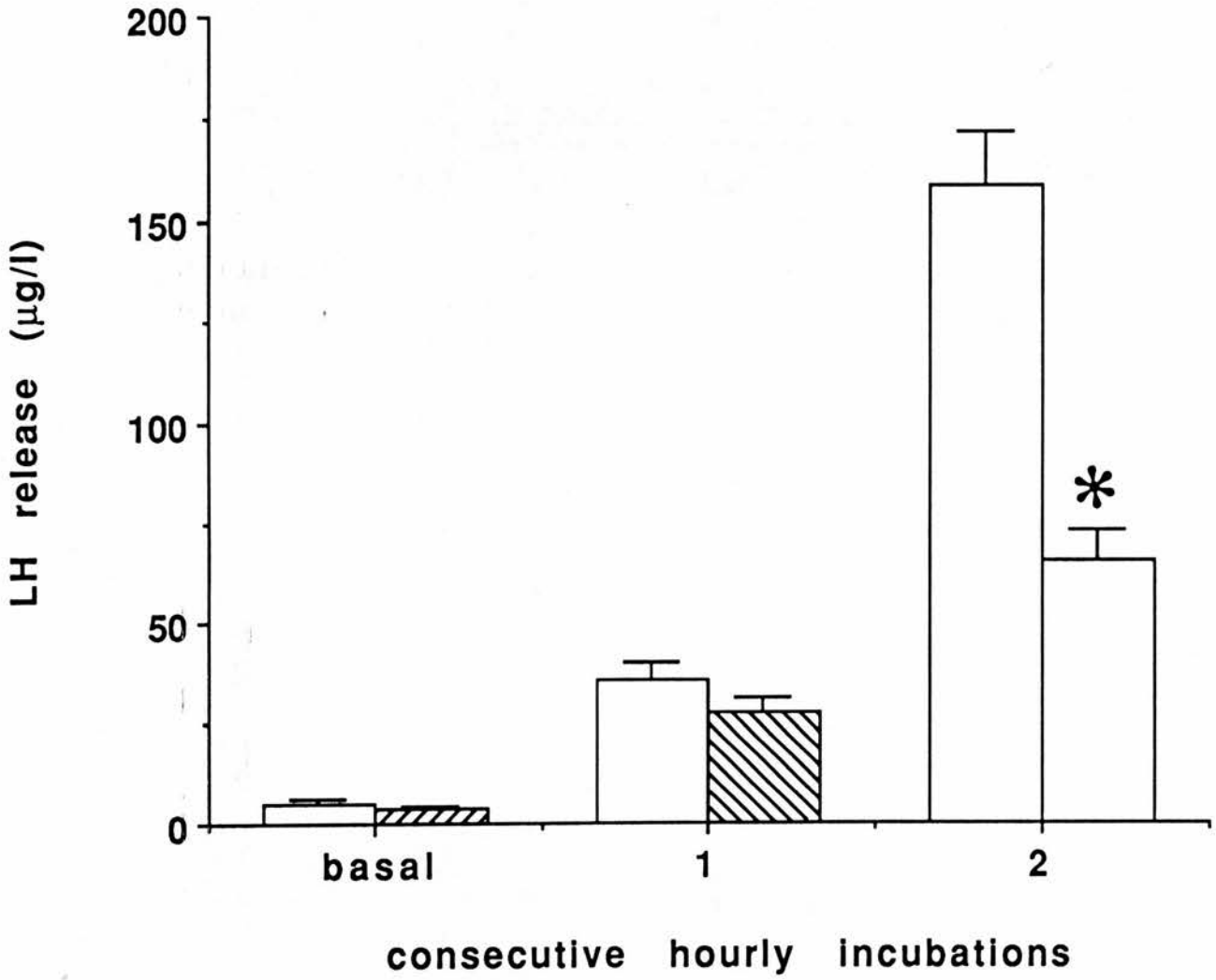
	LH release (μg/l)		
	basal h	1st h	2nd h
no drug			
LHRH	4.9 ± 1.4 (5)	33.9 ± 6.6 (5)	123.3 ± 7.4 (5)
LHRH + ETYA (10μmol/l)	3.6 ± 0.1 (4)	53.2 ± 14.9 (4)	171.1 ± 33.9 (4)
LHRH + ETYA (30μmol/l)	8.5 ± 1.2 (4)	36.3 ± 6.0 (4)	*12.7 ± 4.2 (4)
LHRH + NDGA (10μmol/l)	6.0 ± 3.1 (4)	32.0 ± 2.6 (4)	136.8 ± 5.9 (4)
LHRH + NDGA (30μmol/l)	3.7 ± 0.4 (4)	*6.3 ± 1.2 (4)	*6.32 ± 3.16 (4)


200
150
100
50
0
LH release (µg/l)
*
basal
1
2
consecutive hourly incubations


FSH release (µg/l)
35
30
25
20
15
10
5
0
basal
1
2
*
consecutive hourly incubations


200
150
100
50
0
LH release (µg/l)
*
*
0
10
100
quinacrine (µmol/l)


250
200
150
100
50
0
LH release (µg/l)
0
10
100
BrPheBr (µmol/l)
*
*
*
*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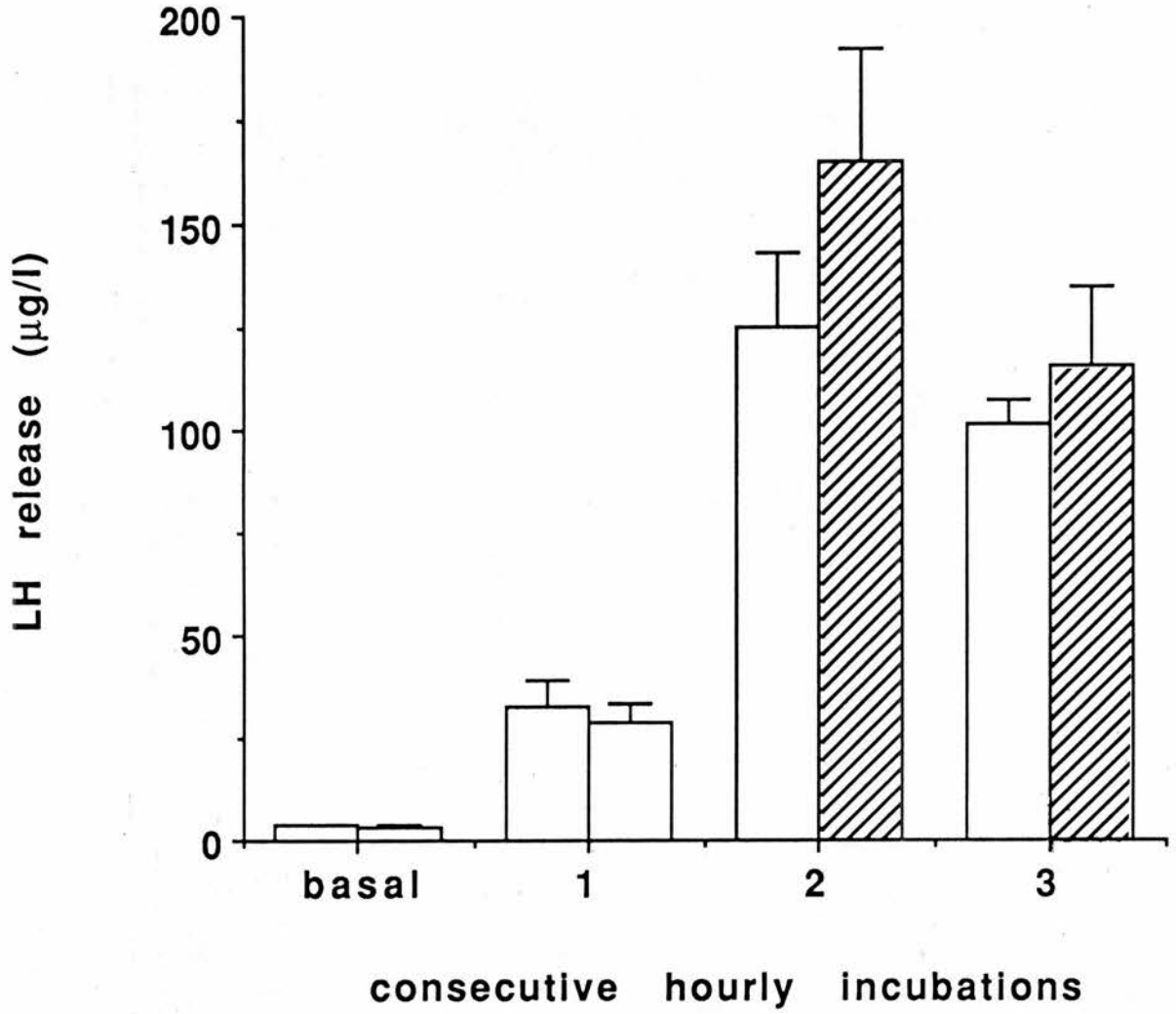
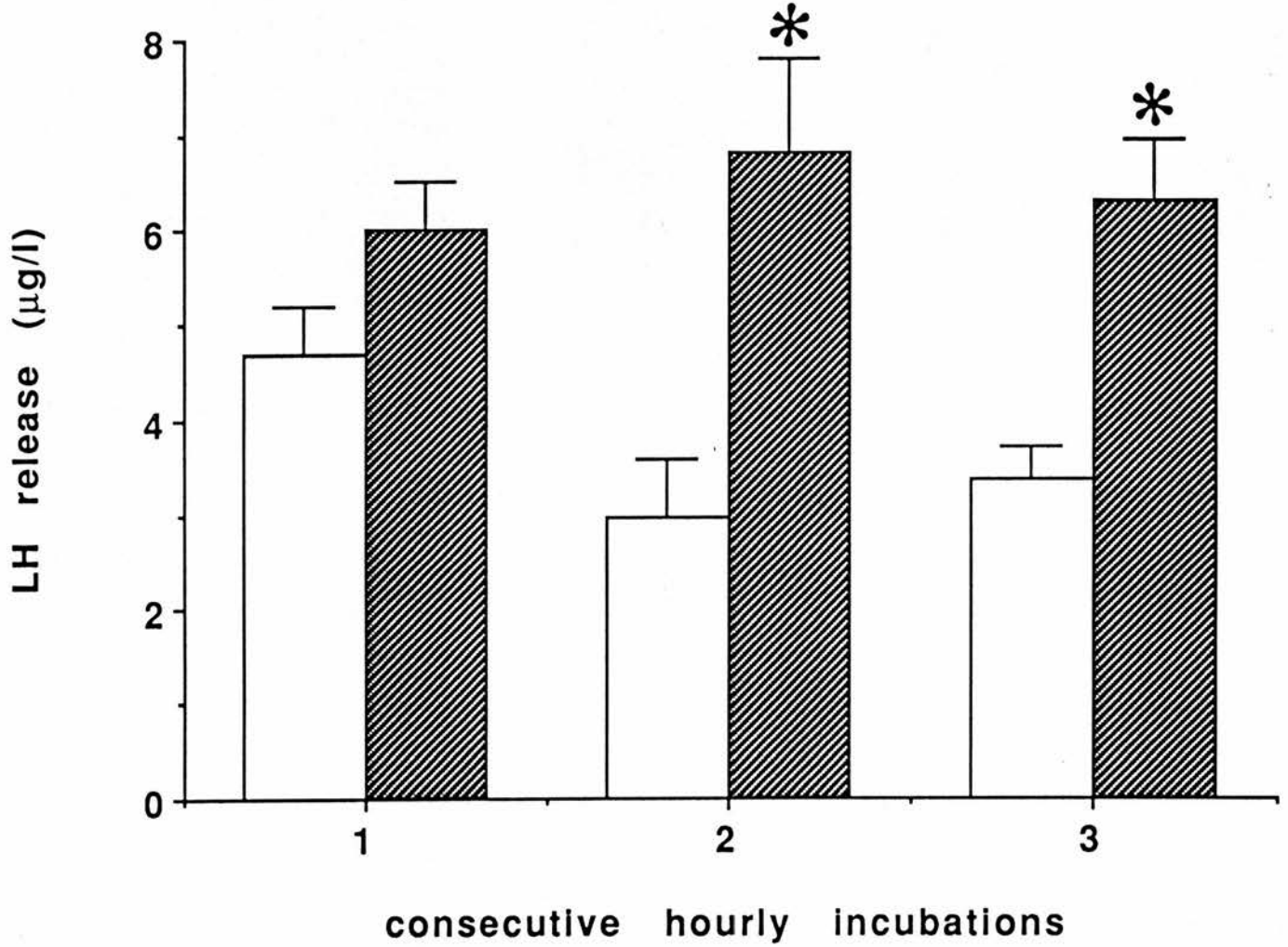
150
100
50
0
LH release (µg/l)
*
basal
1
2
consecutive hourly incubations


200
150
100
50
0
LH release (µg/l)
basal
1
2
3
consecutive hourly incubations


LH release (µg/l)
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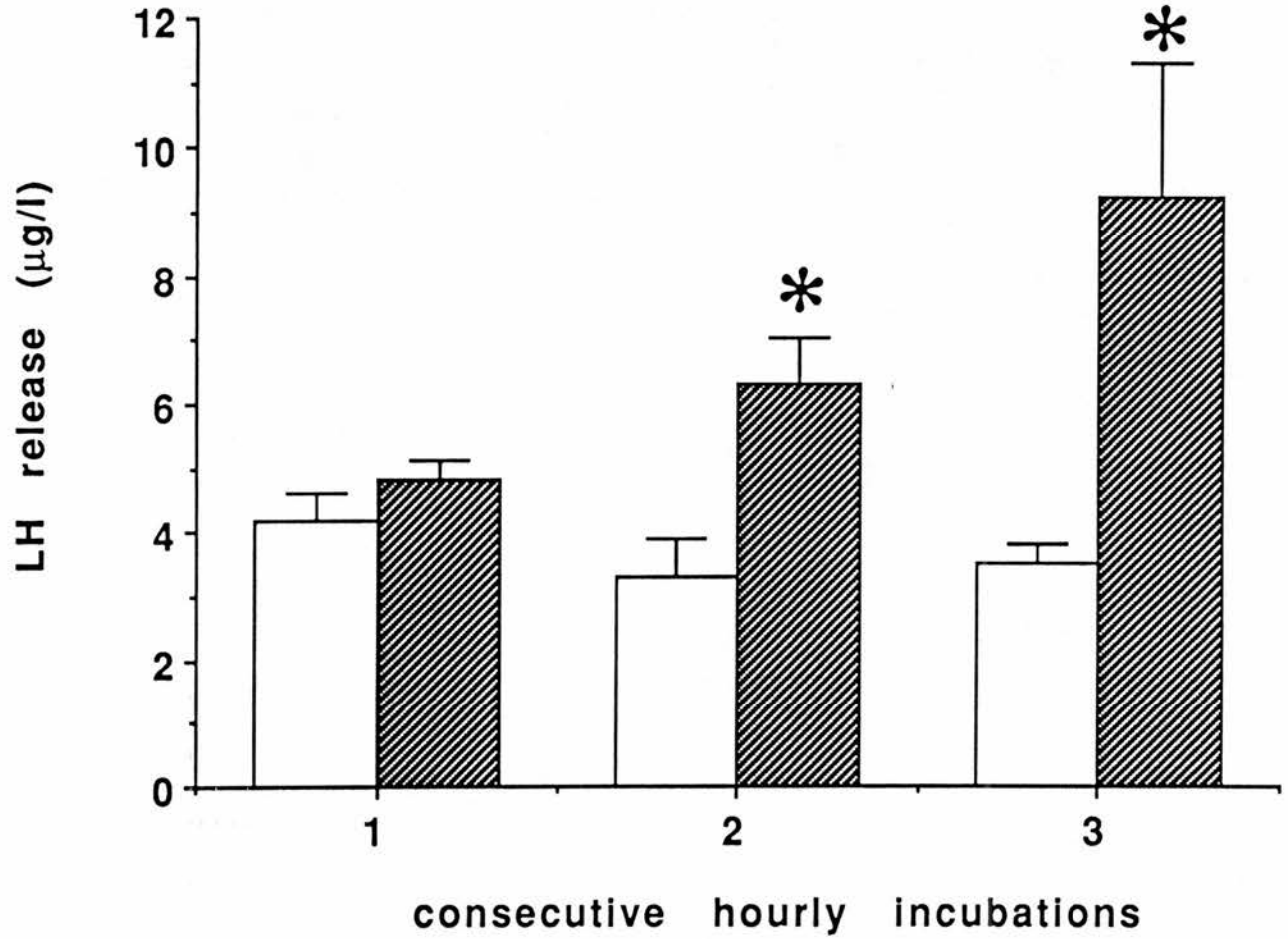
6
4
2
0
*
*
1
2
3
consecutive hourly incubations


LH release (µg/l)
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10
8
6
4
2
0
*
*
1
2
3
consecutive hourly incubations